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Influence of Ectomycorrhiza on exudation of Low Molecular Weight carboxylates in *Pinus sylvestris* L.

By
The Faculty of Environmental Sciences and Process Engineering,
Brandenburg University of Technology Cottbus, Germany
has approved the thesis for the award of
PhD degree in Environmental Sciences

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Date of oral examination: 24th January 2008

Einfluss von Ektomykorrhiza auf die Exsudation von niedermolekularen Carboxylaten unter *Pinus sylvestris* L.

Von
der Fakultät für Umweltwissenschaften und Verfahrenstechnik
der Brandenburgische Technische Universität Cottbus
zur Erlangung des akademischen Grades eines
PhD- Umweltwissenschaften
genehmigte Dissertation

vorgelegt von

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Acknowledgements

I would like to express my sincere thanks to Prof. Dr. Dr. h.c. Reinhard F. Hüttl, Head of Chair of Soil Protection and Recultivation, Brandenburg University of Technology, Cottbus, Germany, for his generosity in accepting me as a PhD student to work under his guidance.

My regards to Dean Prof. Dr. Gerhard Wiegleb, Head of Chair of General Ecology, Brandenburg University of Technology, Cottbus, Germany, for his suggestions as a co-ordinator for seminars of ERM-PhD and my deep thanks to him to accept my request for guidance. My due regards to Prof. Dr. Michael Schmidt, Head of ERM-PhD, Chair of Environment Planning, Brandenburg University of Technology, Cottbus, Germany.

My sincere gratitude to my supervisors, Dr. Uwe B. Schneider, Chair of Soil Protection and Recultivation, Brandenburg University of Technology, Cottbus, and Dr. Thomas Fischer, Central Analytical laboratory (Zentrales Analytisches Labor), Brandenburg University of Technology, Cottbus, in supporting my work to bring to conclusion. I would also like to extend my regards to PD Dr. Dr. Reiner Petzoldt for his suggestions and reading my thesis.

My sincere thanks to Frau Gabi Franke, Frau Regina Müller and Frau Helga Köller for analysis and helpful laboratory tips.

I would like to express my sincere thanks to PD Dr. Udo Bröring, Chair of General Ecology, Brandenburg University of Technology, Cottbus, for indepth discussions on principle component analysis and hints, which were very helpful.

Finally yet importantly, would like to express my indebtness to past and present co-workers at the Chair of Soil Protection and Recultivation for their contributions and suggestions to me, casting a cherishable joy in my life. I would also like to thank Mr. Seth Nii-Annang-M.Sc., for proof reading this thesis.

Finally, I would like to extend my gratitude to all the mentors making it possible for me to be a recipient of DAAD-STIBET matching funds in the earlier phase and Graduiertenstipendium (Graduate scholarship) in the later phase of ERM-PhD studies at Brandenburg University of Technology, Cottbus, Germany.

To *Vaishnovi…*

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Abbreviations

AAS	Atomic absorption spectroscopy
CE	Capillary electrophoresis
CNS	Carbon, Nitrogen and Sulphur
CTAB	Cetyltrimethylammonium Bromide
ECM	Ectomycorrhiza
e.g.	For example
$\overline{G}$	Gini Coefficient
H	Height
HPLC	High Performance Liquid Chromatography
ICPES	Inductively Coupled Plasma Emission Spectroscopy
I.D	Inner Diameter
i.e.	that is
LAR	Leaf Area Ratio
LMW	Low Molecular Weight
MHB	Mycorrhization Helper Bacteria
MOM	Metal Organic Matter
O.D	Outer Diameter
p (statistical)	Level of confidence 95% and significance level ($\alpha = 0.05$)
PCA	Principle Component Analysis
PE	Polyethylene
PEP	Pospho-Enol- Pyruvate

PET	Polyethylene Terephthalate
psi	Pound per square inch (Pressure unit)
PVC	Polyvinyl Chloride
RTD	Root Tissue Density
SRL	Specific Root Length
TEMED	N,N,N',N'-Tetramethylethylenediamine
UV	Ultraviolet
v/v	Volume to Volume
w/w	Weight to weight
Ø	Diameter
α	Significance level

Abstract

Ectomycorrhiza has a strong influence on exudation of both Low Molecular Weight (LMW) carboxylates and on the release of inorganic compounds into the rhizosphere. This process of exudation influences the chemical composition of the rhizosphere soil than the bulk soil. This specific chemical environment which is created in the rhizosphere significantly influences mineralization processes in the soil. Exudation is normally related to activities such as growth and physiological development of plant roots. Root secretions are also comprised of large range of organic and inorganic substances. To a large extent nutrient uptake by plants depends on the release of exudates in the form of LMW carboxylates, which in turn mobilize the required elements suitable for metabolic purposes by forming organometallic complexes. Root exudates influence the rhizosphere soil making it conducive for root proliferation. Exudates are well known for stimulating the metabolic activity of microbial communities in the rhizosphere. Correspondingly, microbes may enhance rhizodeposition by roots.

The main objective of this research focuses on characterization of Low Molecular Weight (LMW) carboxylates in rhizosphere soil solution and their role in plant nutrition. For a better understanding of the characteristics of the root exudates, *Pinus sylvestris* L. tree seedlings, distinguished into non-mycorrhized as well as mycorrhized variants and were grown in petridish rhizotron. To reduce the influences of the unknown processes occurring in the natural environment, working under sterile conditions to much extent was preferred. To achieve maximum recovery of LMW carboxylates, density of the roots per volume in our petridish experiments was increased. Rhizosphere soil solutions were collected with the help of sterile plastic mini suction tubes (Rhizon). The types of exudates analyzed in the rhizosphere soil samples were oxalate/oxalic acid, L-malate/L-malic acid, citrate/citric acid, succinate/succinic acid and lactic acid. For identifying possible organometallic complexes, detection of labile ion concentration of the above LMW carboxylates was done using capillary electrophoresis and high performance liquid chromatography instruments. Growth of individual plants, within the treatment and across the variants was supposed to offer more information with regard to the influence of the ectomycorrhizal symbiosis on LMW carboxylate release. For inducing mycorrhization of the tree seedlings, a single strain of ectomycorrhiza (AM747290) was used to avoid influence of allelopathic interactions when more strains are used (Wöllecke 2001). In order to characterize the impacts of mycorrhization on characterization of LMW carboxylates, the plant morphological parameters, such as, root tissue density (RTD) and specific root length (SRL), along with the above and below ground biomass were measured for mycorrhizal and non-mycorrhized seedlings. The task of accurate measurements included the consideration of excess weight contributed by the quartz sand particles adhered to

roots. Weight corrections were applied during chemical and morphological measurements to increase the accuracy.

Further, the organometallic complexes in the rhizosphere solution were calculated using a geochemical modelling software tool - PHREEQC. Depending on the complex forming abilities of LMW carboxylates present in the rhizosphere, organometallic complexes are formed with the cations. The outcome of this task was a development of a conceptual model to predict and compare the nutrients present in the plant to their availability in the rhizosphere which was supplemented by artificial nutrient supply.

1. Introduction

1.1 General background

Soil solutions provide nourishment for the support of microbes and plants, which in turn assimilate by way of nutrient uptake. Soil solutions also provide a medium in which surface and solution reactions occur, which indirectly impose osmotic stresses on plants and microbes. This in turn helps in transport of materials through the soil (Krauskopf and Bird 1995). Plant roots play an important role by interacting with the microbes causing direct effects on nutrient uptake (Atkinson 1990a). Plant-microbe relationships are interdependent with microbes deriving nutrient from plants and in return, microbes assist in availability of nutrient for plant's growth (Brimecombe et al. 2001). Low organic matter accumulation during reclamation of mineland soils poses a major problem in the mobilization of nutrient for the plants (Schaaf et al. 1999). The processes influenced by roots in the rhizosphere such as secretion of root border cells and root exudates are not well understood (Hawes et al. 2000). The root exudates influence the rhizosphere soil making it conducive for the growth of plants (Marschner 1995). The ability to secrete a vast array of compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots, with nearly 5% to 21% of all photosynthetically fixed carbon being transferred to the rhizosphere through root exudates (Marschner 1995). As a consequence of normal growth and development, a large range of organic and inorganic substances are secreted by roots into the soil, which inevitably lead to changes in its biochemical and physical properties (Rougier 1981). Further, this process causes modifications in soil profile, which is otherwise, known as weathering (Lundström 1994).

1.2 Rhizosphere

The term rhizosphere is derived from the Greek language, meaning the influence of the root on its surrounding, and was first used by Lorentz Hiltner in 1904, in his pioneering work on the "rhizosphere". It was used to indicate the zone of soil where root exudates released from plant roots can either stimulate, inhibit or have no effect on activities of soil microorganisms (Pinton et al 2001a). The rhizosphere is differentiated into: 1) the ectorhizosphere, a soil layer surrounding the roots; 2) the endorhizosphere, a root layer colonized or potentially colonized by microorganisms; and 3) the rhizoplane, is the root surface separating the two areas (Curl and Truelove 1986; Pinton et al. 2001b). The mycorrhizosphere indicates the soil surrounding the root infected by mycorrhizal fungi and explored by fungal hyphae (Curl and Truelove 1986; Pinton et al. 2001b). Thus, rhizosphere is an environment created by the interactions between the mineral soil, root exudates and microorganisms that may either utilize the organic materials released as nutrient

sources or be inhibited by them (Lynch 1990b). In this study, the term rhizosphere focuses on the ectorhizosphere (for non-mycorrhizal variant) and mycorrhizosphere (for mycorrhizal variant). The rhizosphere is the boundary between the plant and the soil community influenced by the presence of the roots, across which movement of water, nutrients and exudates occurs (McCully 1999). Similar to all boundaries, the rhizosphere is complex in character and "probably the most complex environment on earth" (Belnap et al. 2003).

1.3 Root exudates

Root exudation is mainly a passive process existing between the cytoplasm of intact root cells and the external rhizosphere (substrate around the root region) leading to release of products into the rhizosphere (Uren and Reisenauer 1988). Root exudates consist of different fractions, such as, diffusates, excretions and secretions. Diffusates comprise of sugars, amino acids, water, inorganic ions, oxygen, riboflavin etc. and also low molecular weight (LMW) carboxylates belong to this category (Curl and Trueglove 1986). Root exudates are traditionally grouped into low- and high-molecular weight compounds. The majority of root exudates termed as low molecular weight (LMW) compounds consist of amino acids, organic acids, sugars, phenolics and various other secondary metabolites (Neumann and Römheld 1999). Accordingly, high molecular exudates, primarily, include mucilage (polysaccharides) and proteins. LMW carboxylates are common substances found in nature, influencing the metabolic activities or organisms. In soils, LMW carboxylates are also found by way of microbial degradation of organic material (Fox & Comerford 1990). Thus, carboxylates are also nutrients for microorganisms (Lundström 1994; Jones and Darrah 1994). Buurman and van Reeuwijk 1984); Lundström and Giesler 1995) and van Hees et al. (1999), suggested "the importance of LMW carboxylates in the complexation and transport of Al and Fe in forest". Hue et al. (1986); Fox and Comerford (1990); Krzyszowska et al. (1996); van Hees et al. (1999, 2000) and van Schöll et al. (2006), "have identified LMW carboxylates in the soil solution of podzols". For example, "mostly, Al and Fe elements form complexes with the carboxylic acids, such as, citric and oxalic acids, and have high stability constants" (Hue et al. 1986). Also, it has been found that "most of the organic solutes belonging to O horizon have Al" (van Hees et al. 1999, 2000). Due to their complex forming ability and readiness for microbial decomposition (Boudot et al. 1989), carboxylates may play an important role in the podzolization processes. Though these processes lead to biotic development by altering the physical and chemical factors that are limiting (Visser 1985), "exogenously supplied organic matter is needed to start the ecosystem" (Tate 1985). As most root exudates consist of mono-, di-, tri-carboxylates and hydroxycarboxylates (Filius et al. 1997; Jones and Brassington 1998), the present study focuses on investigation of oxalate, citrate, L-malate and lactate as LMW carboxylates. The criteria for selecting these particular ligands were that (i) they are

common secretion products of prokaryotes, fungi, and plant roots, (ii) they are common low-molecular-weight constituents of dissolved organic matter in soil solutions (Baziramakenga et al. 1995; Krzyszowska et al. 1996; Jones 1998), and (iii) they exhibit a wide range of molecular structures that potentially influence their chemical reaction with surface-bound and aqueous-phase metals (Furrer and Stumm 1986).

1.4 Ectomycorrhiza

Mycorrhization in planted tree seedlings, may involve a chemical signalling inducing the growth of fungal hyphae towards the roots of host plant roots (Melin 1954; Horan and Chilvers 1990), but the specificity of signalling or the type of substances involved are not understood so far. Mantle hyphae penetrate between the epidermal cells and cortical cells of the root tips forming a network of mycelium, called as Hartig net (Read 1991). This is the site for exchange of nutrients. The mantle connects to the outside through a network of hyphae to the environment, increasing the absorption surface of the root tips (Smith and Read 1997). Thus, an improved nutrient uptake capacity of ECMs can be due to an increase in the nutrient absorbing surface area of the root system by an extensive extrametrical mycelium (Rousseau et al. 1994). The small hyphal diameter increases the area of the absorbing surface for exploring a large interstitial soil volume and the extramatrical mycelium provides a high affinity for nutrient uptake. It also facilitates solubilization of soil nutrients by the release of proteases, acid phosphatases, and oxidases (Bending and Read 1995). The fungal PEP-carboxylase cycle is important integrator of carbon and nitrogen metabolism, since it supplies C for amino acid skeletons (Wingler et al. 1996; Martin et al. 1998). The predominant forms of N transferred to the host are glutamine, glutamate, and asparagine (Smith and Read 1997). Phosphorus (P) is often present in the soil as insoluble inorganic or organic forms. The plants can only absorb soluble forms of P and their uptake rapidly causes a P depletion zone around the roots. ECM formation increases plant phosphorus content and the ability of the plant to gain phosphorus (Finlay and Read 1986; Perez-Moreno and Read 2000). ECM fungal hyphae form polyphosphates from part of the imported P. Polyphosphates are an important P storage compound and together with orthophosphate, short chain polyphosphates are the predominant form for P transported towards the host plant (Smith and Read 1997). Plant and fungal communities are entities in competitive surroundings and are important for the improvement of nutrient uptake and its adaptation to specific site conditions. Correspondingly, the mycorrhizal fungal strain composition can determine the plant growth and diversity (van der Heijden et al. 1998).

1.5 General approach

For the rhizosphere studies a detailed description of the spatial and temporal variation of soil-root interface would require huge amount of soil samples, causing insurmountable difficulties in integrating results. Thus, for studying process-oriented mineralization-immobilization in the rhizosphere, experimental setups need to be based on manageable microcosms or mesocosms and on simplified approaches that can be compared to the field conditions. An evitable exception of laboratory rhizosphere studies to the field conditions is, however, that – "it is a thermodynamically closed system" (Badalucco and Kuikman 2001).

For the realization of the present rhizosphere study, preliminary experiments of growing *Pinus sylvestris* L. seedlings in three different growth substrates were undertaken for a period of 3-7 months, i.e., a) in silica beads (Ø < 2 mm), b) surface modified silica beads(Ø < 2 mm) and c) quartz sand (Ø < 2 mm). In the silica beads substrate seedlings showed stunted growth (Fig. 1a) whereas vital growth of plants was observed in the quartz sand (Fig. 1b). The pH increased from 6 (in the supplied nutrient solution) to 9.0 in the sampled rhizosphere soil solution. Similar observation of pH increase was also reported by other researchers when they used soda glass beads as growth substrate for their microcosms studies (Sandnes and Eldhuset 2003). For obtaining surface modified silica beads, the silica surface was coated by rinsing with 5% Silan (dichloro-dimethylsilane) reagent for 10 seconds. The silica beads were rinsed twice with hexane, then with methanol and finally, with distilled water (until neutral pH). The silca beads were oven-dried for 1 hr at 70°C (Wenzler et al. 1997). The purpose of using the surface modified silica beads was to increase adsorption of nonpolar organic substances exuded by the plants to enhance selective sampling. The experiment using surface modified silica beads were not successful as the seedlings did not survive, suggesting that, silan had caused a phytotoxic effect. Hence, only quartz sand was selected as a growth substrate for the experiments carried out in this thesis.

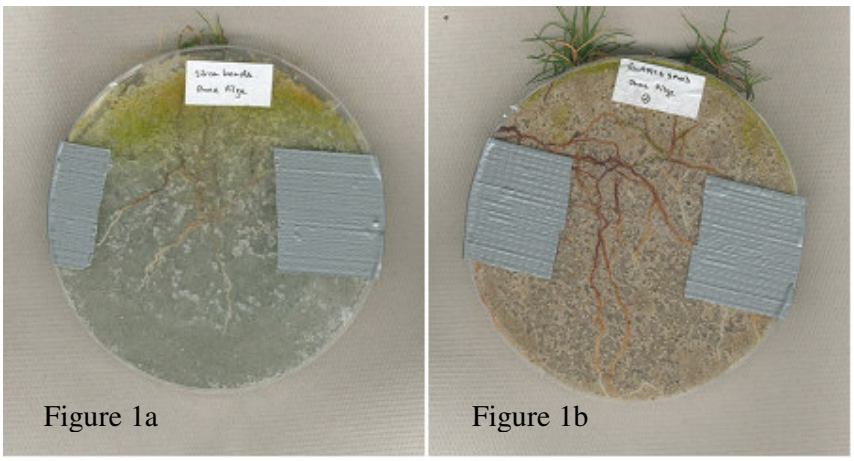


Figure 1. Petridish showing stunted growth in silica beads (Figure 1a) and vital growth in quartz substrate (Figure 1b).

To differentiate between various species of low molecular organic acids it was necessary to develop a working method to analyze such exudates by using High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE). In this study, the comparison of the cations bound to LMW carboxylates in a rhizosphere soil solution was also investigated. Both methods allow detection of 'labile' forms (Göttlein A. 1998) of LMW carboxylates (COO^-), anions (X^-) and cations (M^{n+}) in the rhizosphere soil solution. Therefore, information about the rate of cations that plants can directly take up, as well as, rate of speciated form of LMW carboxylates that plant use to actively influence nutrient recovery and element complexation can be known at the same time. The rhizosphere soil solution was collected using porous plastic mini suction cup tubes (Rhizon).

1.6 Nutrient uptake by plants

The rhizosphere is a complex and heterogeneous environment consisting of exudates, lysates, mucilage, secretions, dead cell materials and carbondioxide; which affect the substrate flow to the roots (Lynch and Whipps 1990). Organic acids and humic substances in soil act as ligands and the resulting complexes can be important in the movement of metals ions (Lindsay 1974). Plant roots modify their environment by way of pH changes, exudation and microbiological activity in the rhizosphere (Mehra and Farago 1994). Bowling (1976) has suggested that there are four links in the uptake chain: a) movement of ions or complexes in the soil to the roots; b) uptake into the root; c) transport across the root to the vascular system; and d) movement towards the shoot. Root nutrient uptake and transport through the roots can occur by (1) diffusion, (2) advection, and (3) by active uptake.

The absorption of elements by roots can be both active or metabolic, and passive or non-metabolic (Mehra and Farago 1994). Active transport is by definition a process in which energy, provided by respiration, is expended in moving ions from a zone of lower to higher electrochemical potential or concentration. Energy demand for ion uptake can be large and can consume as much as 35 % of the total respiratory energy (Marschner 1995). Differences between 'passive or physical' and 'active or metabolic' nutrient adsorption were introduced by Epstein (1960). The fundamental difference between passive and active transport is determined by the description of coupled flow of water, solute, heat, and electrical charge, using the general theory of irreversible thermodynamics. Plant root water uptake is generally considered passive only, although some active water movement may occur by electro-osmosis and other physiochemical mechanisms (Dainty 1963; Slayter 1967). However, it is reported that the distinction between passive and active uptake is not so clear and depends on consideration of driving forces describing the total mass transport. The two different mechanisms lead to transport 'down a gradient' and 'against a gradient', respectively. A large part of the root consists of relatively large and loosely

arranged parenchyma cells with air spaces, collectively known as cortex. The central cylinder of the root or stele contains the vascular system which is responsible for the transport of water and minerals throughout the plant. These tissues contain xylem for conduction of water and nutrients up to the aerial parts, and the phloem conducts the movement of organic material in the opposite direction, i.e. from the leaves towards the roots. The stellar portion of the root is surrounded by a distinct layer of cells, the endodermis, which separates the stele from the cortex. This distinct feature of the endodermis is the Casparian strip or band that surrounds the walls of each cell. The Casparian band is composed of suberised material which forms an impermeable barrier to water and ionic solutes. A typical mesophyll cell wall structure is made up of pectic substances, cellulose and hemicellulose. The aggregate form of cellulose is known as microfibrils. Passive transport occurs in the root's cortex, and is kinetically-controlled by diffusion and mass flow, with ion exchange occurring between solution and the negatively-charged cell walls. Since diffusion across the plasmalemma (membrane between the cytoplasm and cell wall) or the tonoplast (membrane separating the vacuole and cytoplasm) may be severely limited, active transport mechanisms to move specific ions into the cytoplasm, across the plasmalemma, and vacuole, across the tonoplast, are required. Specifically, transport of water and nutrients is impeded by the presence of the Casparian band in the endodermis. The active ion transport across the plasmalemma and tonoplast is driven by specific energy-driven ion carriers or through ion channels embedded in slowly permeable, hydrophilic lipids within the cell membrane. Cell membranes control transport of nutrients from the apoplast (cell walls) to the symplast (cytoplasm and vacuole) and subsequently into the xylem. Transport into xylem can be passive or active and is a problem still to be resolved (Streit and Stumm 1993). Cell membranes capability of transport and its regulation is closely related to their chemical composition and molecular structure. These membranes dominantly consist of hydrophobic polar lipids, which are combined by extrinsic proteins on the outside of the membrane with hydrogen bonds to provide hydrophillic sections. In this way, active ion transport is mediated across the membrane; however, ion movement is by a diffusion type of transport. Alternatively, intrinsic proteins may be integrated into the membrane, allowing movement of hydrated nutrients through small open spaces or voids (< 0.4 nm) (Clarkson 1974; Marschner 1995), such as by ion pumping. In addition, protein channels within the membrane can provide pathways for specific ion movement across the membrane (Marschner 1995). The energy required for active nutrient transport is metabolically driven by reduction of ATP to ADP through ATPases enzymes. This causes transport of ions across membranes from the apoplast to the symplast, from the cytoplasm into the vacuole, or in opposite directions. Specifically, ATP-driven proton pumps provide a major ion pathway through transport of H^+ from within the cell to the apoplast, thereby creating pH and electropotential gradients by which both cations and anions can move across

respective membranes by ion channels or carriers (Marschner 1995). Thus, these proton pumps provide the driving force for energized transport of ions along electrochemical gradients across either the tonoplast or plasmalemma for passive nutrient transport. The active ion transport across the plasmalemma and tonoplast of root cells is driven by specific energy-driven carriers and ion channels. Both mechanisms require the creation of electrochemical gradients across membranes by metabolically driven ion pumps (Bassirirad 2000).

The intrinsic difference in uptake mechanisms between passive and active uptake leads to different nutrient concentrations in soil solution. Specifically, passive nutrient uptake by convective water flow does not alter the soil solution concentration, whereas active uptake reduces the average nutrient concentration in the soil. Active rate of uptake and transport within the plant and its ion-selectivity is regarded as a kinetic process, equivalent to that described by Michaelis-Menten type of kinetics (Epstein 1972). At present there is little information on the relationship between concentration of the complexed species and their uptake rate by plants though some general evidence suggests that the metal complexes in soil must split before uptake occurs, indicating that it is the free ion which is absorbed (Bowie and Thornton 1985).

In this thesis nutrient uptake rate studies are not carried out, rather, a conceptual model is presented showing the assimilated nutrient concentration in relation to the substrate nutrient concentration in the rhizosphere at the culmination of the experiments. The conceptual model focuses on the effect of LMW carboxylates on mobilization of nutrient element in the rhizosphere soil for its uptake in the plant.

1.7 Objectives

The main objective of this thesis was to investigate the types of LMW carboxylate exudates in the rhizosphere of *Pinus sylvestris* and investigate the quantities of exudates released due to mycorrhization

The collected rhizosphere soil solution was analyzed for exudates (LMW carboxylates) using a capillary electrophoresis (CE) and a high performance liquid chromatography (HPLC). LMW carboxylate species analyzed using a CE are having suffix ‘-ate’ and using a HPLC, they are analyzed in acidic forms, having suffix ‘-ic’. The purpose of using both the instruments (HPLC and CE) for analysis of LMW carboxylates was to compensate for the drawbacks occurring in either of the instruments in selective separations or identification of the desired compounds. Also, the cations present as labile ions were analyzed by CE.

As less sample volume is required for analysis by capillary electrophoresis, it is an advantage (Jandik and Gunther 1993).

As to the potential influence of the LMW carboxylate release on the chemical constitution of the rhizosphere, the speciation of organometallic complexes of calcium (Ca), magnesium (Mg), manganese (Mn), aluminium (Al) and potassium (K) with the LMW carboxylates was also taken into consideration. To calculate organometallic complexes of the carboxylates, a geochemical modelling software tool - PHREEQC (Parkhurst 1995) was used.

Based on the modelling results in this study, a conceptual model is developed to compare the nutrient element in the whole plant to the corresponding element availability in the rhizosphere soil solution, which is calculated as cation from a chelate-complex. A comparison is done between the non-mycorrhized and mycorrhized variants.

2. Materials and Methods

2.1 Methodology of Experiments

The present study is based on comparison of root exudates from mycorrhizal and non-mycorrhizal Scots pine seedlings. Experimental conditions were kept as uniform as possible for all treatments with regard to external factors and to maintain homogeneity of plant material. Germinated Scots pine tree seedlings were divided into treatments, as mycorrhizal (n = 75 seedlings) and non-mycorrhizal (n = 50 seedlings) at the beginning of the experiments. The seedlings were grown in petridish rhizotron, each containing five seedlings and were designated as M (mycorrhizal) and NM (non-mycorrhizal) variants. The petridishes, uniform in size, with same substrate were supplied periodical nutrient supplements. The sampling of rhizosphere solution from each petridish was done by using a mini suction sampler (Rhizon) and the sample was analyzed for low molecular weight (LMW) carboxylates. The amount of sample collected varied between 100 µl to 1 ml for each petridish. For identification and quantification of LMW carboxylates, the analysis was done on HPLC and CE.

2.2 Details for cultivating the Scots pine seedlings

2.2.1 Ectomycorrhiza fungal material:

The classification of fungi as given as follows:

Kingdom:	Fungi
Phylum:	Basidiomycota
Class:	Agaricomycetes
Taxon:	Homobasidiomycetes
Accession No.:	AM747290 (Schneider B. and Münzenberger B. 2007)

2.2.2 Agar medium for germinating seeds of Scots pine:

Modified Benecke-Agar medium (Baumann 2004; Tab. A1), was prepared for the germination of Scots pine (*Pinus sylvestris* L.*)* seeds by replacing KH_2PO_4 with $KHSO_4$. The objective of this modification was to grow the seedlings under phosphate stress. The seeds grown in petridishes (Ø 90 mm) germinated into seedlings in 3 weeks.

2.2.3 Agar medium for growing ectomycorrhizal fungi:

The fungi strains were grown on Modified Merlin Norkan Culture Agar (MMNC- Agar) (Marx 1969; Baumann 2004; Tab. A2) medium under sterile conditions. A 4 mm x 4 mm mycelium containing agar plug was used for

inoculating further sterile agar plates. The agar plates were sealed with a sterile tape and were placed in an incubation chamber at constant temperature of 25˚C. Total time taken for fungal growth was about 3-4 weeks.

2.2.4 Germination and growth of Scots pine seedlings:

For the experimental work seeds of Scots pine (*Pinus sylvestris* L.*)* were acquired from Federal Forest Department, Annaburg (Sachsen-Anhalt, Germany). About 40 petridishes coated with agar medium were cultivated with Scots pine seeds for germination. After the growth period of 3 weeks, the petridishes without fungal contamination were selected. The germinated seedlings had a total length of about 6 cm (root and shoot). The petridishes with seedlings were opened in sterile conditions under a microbiological clean bench. From these petridishes five seedlings were transferred to petridishes (Ø = 145 mm) filled with quartz sand which had been wetted with autoclaved distilled water. Shoots of seedlings were made to project out through 3 mm wide notches at the top side of the petridishes for further growth. The above procedure was followed for all the petridishes of the non-mycorrhizal variant. The same procedure as described above was followed for the mycorrhizal seedlings with additional inoculation by introducing ectomycorrhizal strain. This was done by introducing fungal plugs (n = 7) into the petridish next to the root axis. The variants without fungal strain were termed as non-mycorrhizal (NM) and those with fungal inoculation as mycorrhizal (M). Initially, the samples were acclimatized for 3 days by supplying only sterilized double distilled water through a PET syringe with a sterile intra venous needle. The petridishes were kept at a constant temperature of 16˚C/14 hrs at day and 14˚C/10 hrs at night, and 70% humidity in a growth chamber. The light source was by halogen lighting system (IP55 Philips). The experimental setup was done under sterile conditions and was maintained for the rhizosphere part even after transferring seedlings to the green house, where all the petridish rhizotrons were placed in the climate controlled chamber (Fig. 2). The total duration of experiments until completion was 8 months.

Figure 2. Seedlings in petridishes in the temperature controlled incubation chamber.

2.3 Growth substrate (Quartz sand)

Suitable growth medium is required to provide mechanical support for propagation of roots and to provide exchange surfaces for nutrient adsorption and desorption processes. In the study by van Hees et al. (2005), it was recommended to use soil columns (solid support) rather than hydroponic system. This is also important for the ectomycorrhizal mycelium, which needs a solid interface medium to support the growth of hyphae. Thus, in the experiments commercially available quartz sand as growth substrate was used as a solid supporting medium.

For growing plants, quartz sand (fire dried; grain size of < 1 mm; Baumarkt Bahr, Cottbus, Germany) was used as growth substrate. The existing cations in the quartz sand prior to the start of the experiment were estimated by acid digestion procedure (65% (w/w) HNO_3 in closed Teflon digestion vessels under high pressure at 180 ˚C) and were analyzed using an Inductive Coupled Plasma Emission Spectrophotometer (ICPES). Potassium was analyzed using an Atomic Absorption Spectroscopy (AAS). The quartz sand (substrate) elements analyzed were (mg g^{-1}): Ca (0.12), Mg (0.08), Fe (0.48), Al (1.10) and K (0.26).

The quartz sand was sieved into following particle size fractions:

1 mm – 0.63 mm …… 3%

0.63 mm – 0.40 mm …… 15%

0.40 mm – 0.10 mm …… 80%

0.10 mm – 0.063 mm …… 2%

The above fractioning shows that the dominant category is consisting of fine sand. The purpose of the sieving into different size fractions of sand substrate was to provide pore space to allow all the biological activities to occur in the substrate, including the transport of the nutrients, water and oxygen to the root surface (Bengough 2003). The quartz sand substrate used in the experiments was sterilized by autoclaving at 120˚C and at 14.7 psi for 25 minutes. After sterilization, it was stored in drying chamber at 35˚C until further use.

2.4 Nutrient supply

For all the experiments, each of the petridishes was supplied with sterilized Hoagland nutrient solution (Table A3) (Schopfer 1989; Baumann 2004). The nutrient solution was sterilized by autoclaving at 120˚C and at 14.7 psi for 20 minutes. At the beginning of the experiments, the dosage of 5 ml of 30% v/v Hoagland nutrient was supplied every week with 10 ml of sterilized double distilled water supplement provided in between the nutrient dosage. The dosage of nutrient was doubled from 3rd month onwards to 10 ml. The dosage on nutrient was determined on preliminary experimental observations. The nutrient supply and the water supplements were introduced into the petridishes through the notches at the top of the petridishes through a sterile intra venous needle attached to a PET syringe. For the complete experimental duration the total volume of Hoagland nutrient solution supplied was 1 litre.

2.5 Sampling of rhizosphere solution

Sampling of the rhizosphere soil solution was done with the soil moisture sampler known as Rhizon – type MOM (van Hees et al. 2005). The Rhizon sampling tube (mini suction tube) consists of porous plastic material that wets spontaneously and has a standard 0.1 µm pores. The sampler is provided with coextruded tubing (PE inside/PVC outside) of 1 mm internal diameter, a luer connector and has a protective cap. The sampler has a length of 10 cm and a diameter of 2.5 mm, with glassfiber reinforced strengthening wire and 10 cm coextruding tubing (Fig. 3). The sampler having the porous material part is inserted from one side of the petridish through a 3 mm wide hole, which was later sealed with aquarium sealing agent to prevent any leaks. The other end of the mini suction tube can be closed with a stopper after sampling. An adapter is used to fix a PET syringe and rhizosphere soil solution is collected by applying a mild negative pressure by hand. A sampling routine was maintained strictly by alternating nutrient supply, water supply, pausing for 2 days and then the rhizosphere solution (sample) was collected. Again the nutrient cycle was continued by replenishing nutrient solution soon after sample collection. The rhizosphere soil solution sample

of 100 µl to 1 ml was transferred to an ependorf sterile tube and was stored at -10˚C in freeze compartment of refrigerator till further analysis. Dissolved organic and inorganic matter does not absorb on the soil moisture sampler (company's specifications).

The Rhizon – type MOM moisture sampler was supplied by UP Umweltanalytik System, Cottbus, Germany.

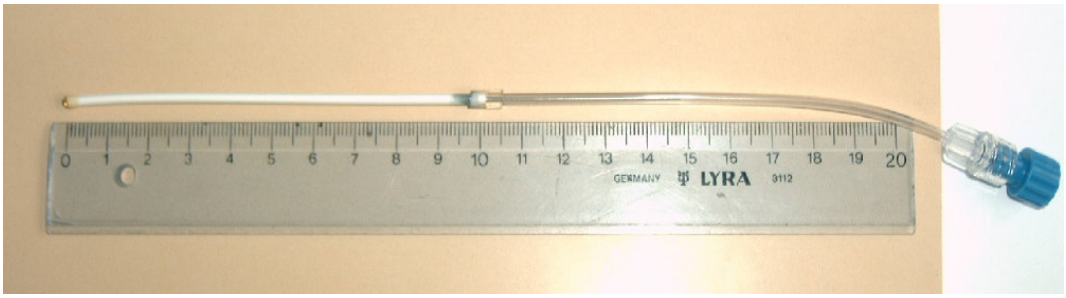


Figure 3. The Rhizon – type MOM moisture sampler used for sampling of rhizosphere soil solutions.

2.6 Procedures for collection of plant material and parameters analyzed

After 8 months of the experimental duration, the plants were harvested. The petridish casing was separated out and the mini suction tube was carefully pulled out as to have minimal destruction to the roots. The contents of petridish containing the quartz sand substrate and plants were transferred into a wash tray. Under cold-water spray, the substrate was washed off. When required a fine painting brush was used while suspending the roots under water in the wash tray for the removal of root adhered quartz sand substrate. The entangled roots of the individual plants were separated out in the wash tray filled with water, by hand. The living plant material and debris (sloughed off root tips) was determined for total biomass. Each individual plant (complete), was stored in a cool place (usually refrigerator) by placing the plant in a PET bottle filled with cool water. Generally, the time for storage was only for few hours to 1 day, until further processing of sample.

The harvested plants were analyzed for elements, such as, calcium, magnesium, sodium, manganese, iron, phosphorus and aluminium using an Inductive Coupled Plasma Emission Spectrophotometer (ICPES). Potassium was analyzed using an Atomic Absorption Spectroscopy (AAS). The total carbon, nitrogen and sulphur contents in plant tissues were analyzed using a CNS - total element analyzer.

2.7 Plant Morphological analysis

After the fresh plant root material was cleared of substrate by washing with spray of water, the individual saplings were checked for mycorrhized root tips in mycorrhized variant under normal dissecting microscope (Olympus SZX9) supported by a camera for capturing pictures and storing them on computer with the help of the software, analysis 3.2 (Build 659), provided by Soft Imaging System GmbH, Germany. The root tips were visually estimated for the abundance of mycorrhizae in percent classes to the total amount of short roots (Fig. 4a) (Giovanetti and Mosse 1980; Dahlberg 1990). The shoot part was separated by cutting at the lower end, i.e., to the starting point of the roots, and further, into stem (Fig. 4b) and leaves (needles) (Fig. 4c). A vernier calliper was used to measure the diameter of stems. For determining the total root length and volume, the roots were cut into short pieces to avoid overlapping during image scanning. The measurement of total root length, average root diameter, root volume, stem height and leaf surface area was by processing the images scanned (400 dpi) with imaging software provided by WinRhizo, Regent technologies, Quebec, Canada. (see Fig. 4a, b, c). The applied scanning resolution of 400 dpi was also preferred for routine analyses with WinRHIZO by Bouma et al. (2000) and Guay (1996) rather than higher ones.

(a) Leaves (Needles)

(b) Stem

(c) Roots

Figure 4. Root, Stem and needles of mycorrhizal seedlings separated for WinRhizo measurements.

2.8 Chemical analysis

For the chemical analysis of the plant tissue and rhizosphere soil solution chemicals were purchased from MERCK (Darmstadt, Germany), SIGMA (Steinheim, Germany) or were supplied by VWR, Germany.

2.8.1 Wet digestion and dry ashing

For the determination of the elements, the plant samples were dried at a constant temperature at 65˚C for 48 hrs in drying chamber under air circulation to constant weight. This procedure of drying at 65˚C ensures that, biological activity is stopped and loss in biomass due to respiration is avoided (Engels et al. 2000). Dried samples were grounded by means of mortar and pistil, to a fine consistency for wet digestion. In wet digestion procedure, the dried plant material is digested in 65% (w/w) HNO_3 in closed Teflon digestion vessels under high pressure at 180˚C.

The excess quartz sand adhered to the root material contributes to the erroneous dry weight. Hence, root material of individual plants was estimated for the quartz sand weight correction. For this purpose, after wet digestion, the concentrate was filtered through an ash-less filter paper (Schleicher & Schuell Ø 70 mm; α-cellulose 95%; ash content 0.008%) and the filtrate of individual plant root material was collected as an aliquot. The undissolved quartz sand particles were left behind on the ash-less filter paper. The aliquot was analyzed for total elements using an ICPES or AAS as required.

The weight of adhered quartz sand to the roots was determined for weight correction by dry-ashing the ash-less filter paper. For this, the ash-less filter paper was placed in a ceramic crucible and was subjected to combustion at 850˚C in a muffle furnace for 4 hours. The non-combustible quartz sand particle residue that was left behind was weighed. Thus, the excess quartz sand weight correction was applied for measuring biomass and for analysis of nutrients in root accurately.

2.8.2 Inorganic cation Analysis by ICPES

Total element concentration of Ca, Mg, Na, P, Al, and Fe were analyzed using an Inductively Coupled Plasma (ICP) Emission Spectroscopy or also known as ICPES. It is an atomic emission technique where atoms and ions present in the sample are excited in high temperature gas plasma. The inductively coupled plasma provides very high temperature in the range of ~8000K. At this high energy, almost all the atoms present in the sample are excited.

ICPES consists of a nebulizer for sample injection purposes, a radio frequency generator producing oscillating current in a coil that wraps around a quartz tube creating a magnetic field. This magnetic field causes the oscillating current in the

ions and electrons in the supporting gas, i.e., Argon, to collide resulting in the generation of high temperature plasma of 8000K-10000K. When the sample is injected into this high temperature plasma, it emits a distinct emission passing through a monochromator, to a detector and ultimately to a recording source. Each individual spectrum of the element is unique which is compared to a standard emissions spectrum and is calculated based on intensity of the signal.

The ICPES instrument used for the analysis was PU UNICAM 701. For the analysis by ICPES an aliquot of 5-10 ml was required.

2.8.3 Inorganic cation Analysis by AAS

The flame Atomic Absorption Spectroscopy (AAS) instrument is used for the analysis of potassium (K). In AAS, a wavelength specific to an element is absorbed and detected from a beam of light passing through a cloud of atoms of the element. The light source is provided by a hallow cathode lamp specified for the element to be analyzed.

The flame AAS instrument used for the analysis was Solar 932 – UNICAM.

2.8.4 Dry matter analysis by CNS

For the analysis of carbon, nitrogen and sulphur a CNS elementary analyzer was used (Elementar Vario El). For the measurement up to 5 mg ground dry plant material is placed in a tin capsule and dropped into a heated glass combustion tube (1150°C) containing Tungsten (IV) oxide granules as an oxidation catalyst. A pulse of pure oxygen is introduced into the Helium gas stream, which flows continuously through the combustion tube. The timing of the O_2 pulse and tin capsule introduction is such that, a flash combustion of the sample occurs. Then the combustion products pass through a heated (850 °C) reduction furnace containing a copper wire in which NO_2 reduces to N_2. The water vapour removal happens while the products pass through a moisture trap containing P_2O_5. After this process, the remaining combustion products, i.e., CO_2 and SO_2, are separated by flowing through the heated trap columns in a step-wise procedure. The respective concentrations of N, C and S are determined as N_2, CO_2 and SO_2 using a thermal conductivity detector.

The C, N and S data obtained was corrected for the excess dry weight contributed by quartz sand adhered to roots by processing the CNS values using MS-Excel worksheet.

The quartz weight correction was applied to the initial sample weight taken for C, N and S analysis:

$$M_2 = (X_1/X_2) \cdot M_1 \qquad \ldots\ldots\ldots\ldots\ldots\ldots 1$$

where,

M_2 (in mg) = corrected weight of sample taken for C, N, S calculations;

X_1 (in mg) = initial weight of sample (plant material + quartz sand) taken for wet-ashing;

X_2 (in mg) = final weight of quartz sand obtained after dry-ashing;

M_1 (in mg) = sample weight taken for C, N, S analysis.

The formula used in calculating %N was as follows:

$$\%N_{\text{after corr.}} = (M_1/M_2) \cdot \%N_{\text{before corr.}} \qquad \ldots\ldots\ldots\ldots 2$$

where,

$\%N_{\text{after corr.}}$ = corrected percentage N in sample (plant material);

M_1 (in mg) = sample weight taken for C, N, S analysis;

M_2 (in mg) = corrected weight of sample for C, N, S calculations;

$\%N_{\text{before corr.}}$ = percentage N in sample (plant material + quartz sand) analyzed on CNS analyzer.

The formula used in calculating %C was as follows:

$$\%C_{\text{after corr.}} = (M_1/M_2) \cdot \%C_{\text{before corr.}} \qquad \ldots\ldots\ldots\ldots 3$$

where,

$\%C_{\text{after corr.}}$ = corrected percentage C in sample (plant material);

M_1 (in mg) = sample weight taken for C, N, S analysis;

M_2 (in mg) = corrected weight of sample for C, N, S calculations;

$\%C_{\text{before corr.}}$ = percentage C in sample (plant material + quartz sand) analyzed on CNS analyzer.

The formula used in calculating %S was as follows:

$$\%S_{\text{after corr.}} = (M_1/M_2) \cdot \%S_{\text{before corr.}} \qquad \ldots\ldots\ldots\ldots 4$$

where,

$\%\ S_{after\ corr.}$ = corrected percentage S in sample (plant material);

M_1 (in mg) = sample weight taken for C, N, S analysis;

M_2 (in mg) = corrected weight of sample for C, N, S calculations;

$\%S_{before\ corr.}$ = percentage S in sample (plant material + quartz sand) analyzed on CNS analyzer.

2.8.5 Analysis of rhizosphere soil solution for Low Molecular Weight (LMW) carboxylates by Capillary Electrophoresis (CE) and High Performance Liquid Chromatography (HPLC)

2.8.5.1 Analysis by Capillary Electrophoresis (CE)

The collected rhizosphere soil solution was analyzed for exudates (LMW carboxylates), such as, oxalate, L-malate, citrate and succinate. Also, labile or free cations, such as, Na^+, Ca^{2+}, Mg^{2+}, K and $Fe^{2+/3+}$, present in the rhizosphere soil solution were analyzed using a CE. The capillary electrophoresis instrument used was P/ACE^{TM} MDQ Glycoprotein System (Beckman Coulter, USA). The software used was 32KARAT provided by Beckman Coulter. The capillary is normal fused-silica capillary from Beckman, cut to 57 cm total length/50 cm effective length to detector with 75 µm I.D and 375 µm O.D. For the purpose of analysis using a capillary electrophoresis (CE) instrument, methodology developed by Göttlein and Blasek (1996) was modified, making it suitable for investigating rhizosphere soil solutions in our experiments.

The basic instrumental configuration of capillary electrophoresis (CE) consists of a fused-silica capillary with an optical viewing window, a controllable high voltage power supply, two electrode assemblies, two buffer reservoirs, and an ultraviolet (UV) detector. The ends of the capillary are placed in the buffer reservoirs and the optical viewing window is aligned with the detector. After filling the capillary with buffer, the sample can be introduced by dipping one end of the capillary (injection side) into the sample solution and applying pressure or vacuum as required. The separation technique used in our experiments was capillary zone electrophoresis (CZE), also known as free solution capillary electrophoresis. In this type of technique, depending on two types of electrokinetic vectors, separation is achieved, i.e., 1) electrophoretic vector (EP) and 2) electroosmotic flow (EOF) vector. Electrophoretic (EP) vector depends on the charge/ionic radius ratio causing the mobilities of analyte ion and electroosmotic flow (EOF) vector depends on the polarity and magnitude of the charge on the capillary walls.

During analysis of LMW carboxylates, the negative electrode is at the injection side and the positive electrode is at the detection side. To reverse the polarity of the capillary tube separation buffer containing CTAB is used. The capillary wall polarity is changed from negative to positive charges causing EP movement of analyte towards the detector side. The EOF is also towards the positive electrode. Thus, the net movement of the anions is towards the detector side and can be separately analyzed. The details of the buffer method used are given in table 1.

Table 1. Description of separation buffer method (modified) used for analysis of LMW carboxylates by CE.

Separation buffer	1,2,4,5 Benzene tetra carboxylic acid (Pyromelitic acid) - 2.15 mM; TEMED - to adjust pH to 8; CTAB - 2.15 mM
Rinse step 1	Regenerator solution A (Beckman Coulter) rinse for 1 minute
Rinse step 2	Double distilled water rinse for 2 minutes
Rinse step 3	Run Buffer rinse for 2.5 minutes
Run voltage	21 kV, 0.18 ramp, Reverse polarity
Injection Method	Vacuum 1.0 psi , 5 seconds with sample diluted in run buffer (if required)
Runtime	15 minutes
Detection Wave length	280 nm UV
Data rate	1 Hz
Filter	Normal
Peak width	6 - 25
Absorbance signal	Indirect

During analysis of cations the positive electrode is at the injection side and the negative electrode is at the detection side. Thus, capillary wall carries the positive charges causing an EP movement of analyte towards the detector side. Correspondingly, the EOF moves in the opposite direction, i.e., towards the positive electrode. As the EP vector velocity is greater than the EOF vector velocity, cations move towards the negative electrode and are detected. The details of the buffer method used are given in table 2.

Table 2. Description of separation buffer method (modified) used for analysis of cations by CE.

Separation buffer	Metol (4-(Methylamino phenol sulphate) - 2.15 mM; Ascorbic Acid - 0.8 mM; 18 Crown 6 - 2.15 mM
Rinse step 1	20 mM run buffer rinse for 2 minutes
Rinse step 2	Double distilled water rinse for 2 minutes
Rinse step 3	Run Buffer rinse for 2.5 minutes
Run voltage	21 kV, 0.18 ramp, Normal polarity
Injection Method	Vacuum 1.0 psi , 5 seconds with sample diluted in ¼ molar run buffer (if required)
Runtime	10 minutes
Detection Wave length	220 nm UV
Data rate	2 Hz
Filter	Normal
Peak width	6 - 25
Absorbance signal	Indirect

2.8.5.2 Analysis by High Performance Liquid Chromatography (HPLC)

For comparative purposes the analysis of LMW carboxylates originating from root exudation was also carried out by HPLC. In this specific case for measurements we applied the HPLC instrument of Gynkotek (Gynkosoft, GmBH) for detection of oxalic, L-malic, citric, succinic and lactic acids. The HPLC separation column was purchased from Phenomenex. Further methodological details are given in table 3.

Table 3. Description of buffer method used for analysis of LMW carboxylates by HPLC.

Column details	Synergi 4µ Hydro-RP C18
Dimensions	75 x 4.6 mm, 4 µm
Flow rate	700 µl/min
Temperature	Room temperature
Detector	UV 205 nm/UV 220 (DAD-UVD 340S)
Dimensions	75 x 4.6 mm, 4 µm
Flow rate	700 µl/min
Mobile phase buffer	20 mM Potassium di-hydrogen phosphate ($KH_2PO_4^{3-}$) adjusted to pH 2.9 by $H_3PO_4^{3-}$

2.9 Calculation of organically complexed cations in the rhizosphere solutions using PHREEQC

For the calculation of cation complexes formed by exudates released in the rhizosphere, a geochemical simulation software PHREEQC was used. In order to assess the influence of such LMW carboxylates on the mobilization/immobilization of cations in the rhizosphere solution, it was hypothesized that the concentration of the resulting fraction of labile-unbound-cations would highly correlate with respective nutrient concentrations in plant tissues.

The concentrations of cations entered into the program for calculations were Na, Ca, Mg, Mn, Al and K found in the nutrient solution (Hoagland nutrient solution) supplied to the seedling and the pH value entered was the measured pH in the petridish rhizotrons. In the program, LMW carboxylate concentrations of oxalate, malate (L-malate) and citrate are obtained from experiments. In the program oxalate concentration is made to adjust implicitly to charge balances by a using a program keyword ‘charge’.

The PHREEQC output gives the possible complexes calculated as molality (Appendix 6). As molality is expressed as number of moles of solute in kilogram solvent, molality is converted to concentration as **mg kg^{-1}** for use in this thesis. From the molality of the ligand metal complexes, the concentration of individual cations were calculated.

According to Uren and Reisenauer (1988) dissociation of citric acid, (H_3X) may proceed in steps as:

I) $H_3X = H_2X^- + H^+$;
II) $H_2X^- = HX^{2-} + H^+$ or
III) $HX^{2-} = X^{3-} + H^+$

The above steps (I, II and III) depend on the pH and the availability of the cations. At various stages of dissociation of other organic acids in the rhizosphere, they can also be available as X^{2-}, X^- etc., for formation of organometallic complexes.

For example, when citrate (H_3X) is taken as an organic acid, the forms of organometallic complexes obtained will be as follows:

a) $M^{n+} + H_2X^- \longrightarrow MH_2X^+$ {Where M^{n+} represents Ca^{2+} & Mg^{2+}}

$\therefore CaH_2Citrate^+, MgH_2Citrate^+$ (see Appendix 6)

b) $M^{n+} + HX^{2-} \longrightarrow MHX$ {Where M^{n+} represents Ca^{2+} & Mn^{2+}}

$\therefore$ CaHCitrate, MnHCitrate (see Appendix 6)

c) $M^{n+} + X^{3-} \longrightarrow MX^-$ {Where M^{n+} represents Mn^{2+} & Al^{3+}}

$\therefore$ AlCitrate, $MnCitrate^-$ (see Appendix 6)

2.10 Relationship between nutrient uptake in plant to the nutrient availability in the rhizosphere soil solutions

According to Hipp and Thomas (1968), it was shown that mostly the analysis of leaf or tissue correlate with soil tests. This would imply that plant tissue analysis reflects conditions of nutrient availability in the soil. Further, it is proposed by Kovacevic and Vukadinovic (1992) that the relationship between nutrient concentration in the plant and nutrient availability in the soil generally follows an asymptotic curve of nutrient uptake, which is controlled by the growth rate of plants and is related to plant's physiological requirements. Sometimes high plant growth rates are associated with high nutrient uptake rates and vice versa (Mengel and Kirkby 2001). When plants starve from a particular nutrient deficiency, they take up the lacking nutrient at a high rate when exposed to increasing concentrations of the nutrient in the solution (Mengel and Kirkby 2001). To show small differences in the plant nutrient concentrations despite the marked changes in the critical nutrient level in the soil solution, an illustration (Fig. 5) was used by Kovacevic and Vukadinovic (1992).

This illustration was adapted as a conceptual model (Fig. 5), to study the relationship of cations formed as organometallic complexes (with the LMW

carboxylates) in the rhizosphere soil solution, to the corresponding nutrient status in the whole plant.

In the conceptual model the nutrient concentration in plant is expressed on a vertical (y-axis) and the corresponding nutrient concentration in the rhizosphere soil solution is indicated by the horizontal line (x-axis). The upper dotted line (left side) in the illustration extends into the nutrient status curve (right side) in the conceptual model. The nutrient status curve is a fitted logarithmic curve derived from the nutrient concentrations in the plant.

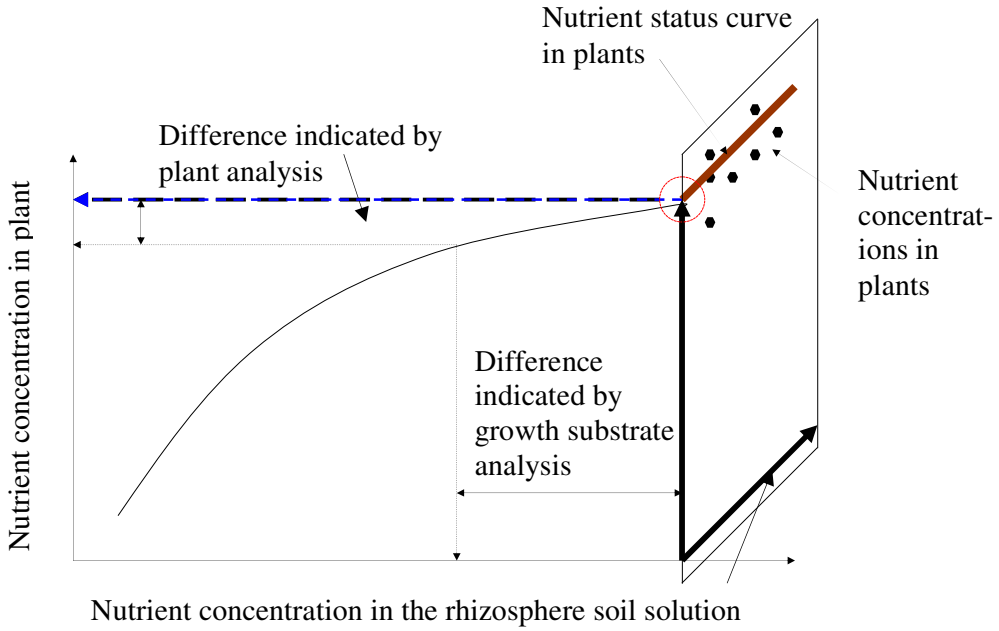


Figure 5. Relationship between nutrient concentration of soil solution and the corresponding nutrient concentration in the plant (adapted from Mengel and Kirkby, 2001).

2.11 Statistics

For the statistical computation of the experimental data, SPSS 11.0 was used. The effect of LMW carboxylate exudates between the non-mycorrhized and mycorrhized seedlings were determined by one-way ANOVA. The confidence level of 95% and significance level ($\alpha = 0.05$) was maintained through out the statistical calculations.

To know the effectiveness of mycorrhization of *Pinus sylvestris* seedlings as a determining factor causing the variations, Principle Component Analysis (PCA) was done. Principle component analysis is based on correlation matrix of morphological and chemical parameters measured. For this purpose experimental data from MS-Excel worksheet is imported into CANOCO software, using the

WCanoImp feature. The choice of axes for explanation of variation with in the variants depends on the maximum value (Eigenvalue) corresponding to the axes (Appendix 7). Hence, in the experiments a total variation of 86.5% can be explained by taking into account the horizontal axis (PC 1, 66.9%) and the vertical axis (PC 2, 19.6%). For the Principle Component Analysis (PCA), the software used was CANOCO® (CANOCO 4.5, Microcomputer Power, Ithaca, USA).

3. Results

3.1 Size distribution and survival of pine seedlings in petridish rhizotron experiments

To evaluate the influence of treatment of mycorrhization on the release of LMW carboxylates in *Pinus sylvestris* L. seedlings, the experiments were started with 15 petridishes having 75 seedlings (5 seedlings per petridish) that were inoculated with ectomycorrhizal fungi and 10 petridishes having 50 seedlings (5 seedlings per petridish) without any fungi. As it was reported that because of mycorrhizal infections, reductions in relative growth rate would occur (Ingestad et al. 1986) leading to loss of specimen. Hence more number of petridishes having mycorrhized seedlings was opted. At the end of 8 months of experimental duration the total number of surviving seedling in the experiments were counted. The total number of non-mycorrhized (NM) seedlings counted were 41 out of 50 (Fig. 6a) and the total number of mycorrhized (M) seedlings counted were 56 out of 75 (Fig. 6b). In two petridishes of non-mycorrhizal experiment only 3 seedlings were found, i.e., 60% and in remaining eight petridishes there were 4 to 5 seedlings, i.e., 88%.

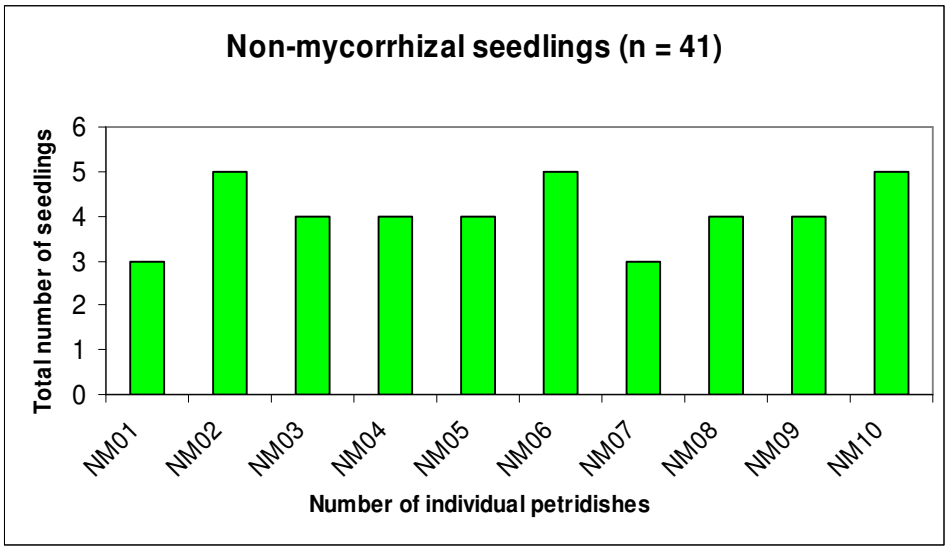


Figure 6a. The number of seedlings found in non-mycorrhizal (NM) experiments (n =10).

Similarly, the number of seedlings in the mycorrhizal experiment was tabulated. For one petridish there was only 1 seedling, i.e., 20%. For five petridishes there were 3 seedlings, i.e., 60% and for remaining nine petridishes there were 4 to 5 seedlings, i.e., 89%. Though, in one petridish with 1 seedling was contributing only 20% in comparison to others, this petridish was not excluded and was investigated for differences in biomass and element distributions with other individual petridishes.

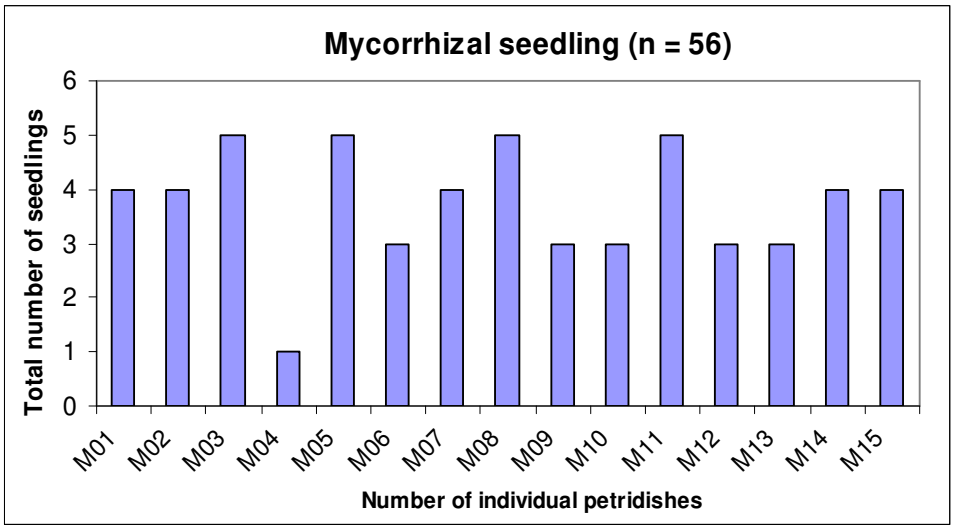


Figure 6b. The number of seedlings found in mycorrhizal (M) experiments (n =15).

Depending on the difference between the number of seedlings at the start and end of the experiments, the overall survival percentage recorded for non-mycorrhizal seedlings was 82% (Tab. 4a) and for the mycorrhizal seedlings was 75% (Tab. 4b).

Table 4a. Rate of survival % of non-mycorrhizal seedlings after 8 months of growth.

Variant	Number of plants before the start of the experiment	Number of plants at the end of the experiment	Survival percentage
NM01	5	3	60%
NM02	5	5	100%
NM03	5	4	80%
NM04	5	4	80%
NM05	5	4	80%
NM06	5	5	100%
NM07	5	3	60%
NM08	5	4	80%
NM09	5	4	80%
NM10	5	5	100%
n =	50	41	
		Mean	**82%**

Table 4b. Rate of survival % of mycorrhizal seedlings after 8 months of growth.

Variant	Number of plants before the start of the experiment	Number of plants at the end of the experiment	Survival percentage
M01	5	4	80%
M02	5	4	80%
M03	5	5	100%
M04	5	1	20%
M05	5	5	100%
M06	5	3	60%
M07	5	4	80%
M08	5	5	100%
M09	5	3	60%
M10	5	3	60%
M11	5	5	100%
M12	5	3	60%
M13	5	3	60%
M14	5	4	80%
M15	5	4	80%
n =	75	56	
		Mean	**75%**

To shed indepth information on the weight distribution of the plants, they were separated into i) above ground parts, such as, stem and leaves (needles); and ii) below ground part as roots. This procedure was followed for non-mycorrhizal experiment (Fig. 7a) and mycorrhizal experiment (Fig. 7b). Atleast, one of the seedlings in each petridish has non-uniform growth, having large root mass (≥20g dry wt.) in comparison to other co-individuals in non-mycorrhizal experiment (Fig. 7a). Similarly, in the mycorrhizal experiment (Fig. 7b), atleast one of the seedlings has large root mass (≥20g upto ≥40g dry wt.). Correspondingly, when root mass was more, the above ground mass for that individual was also more.

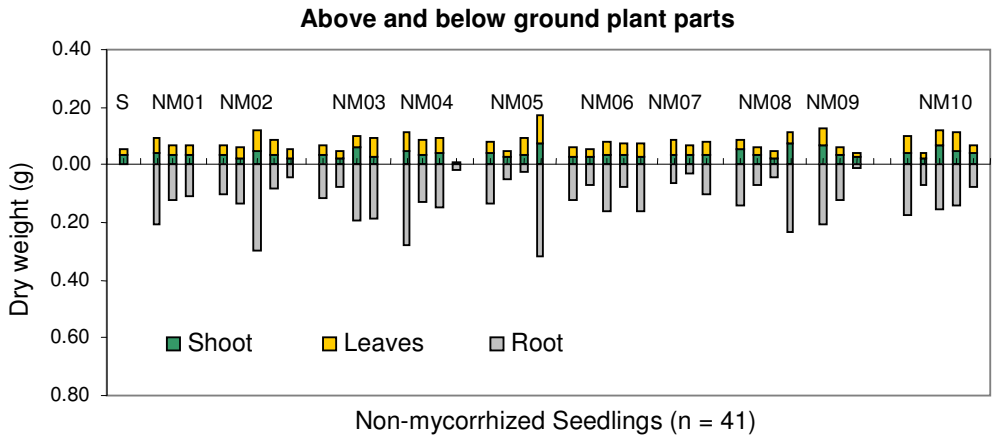


Figure 7a. Shoot and root dry weight (in g per seedling) for individual non-mycorrhizal (NM) (n = 41). S = Seedling before the experiment.

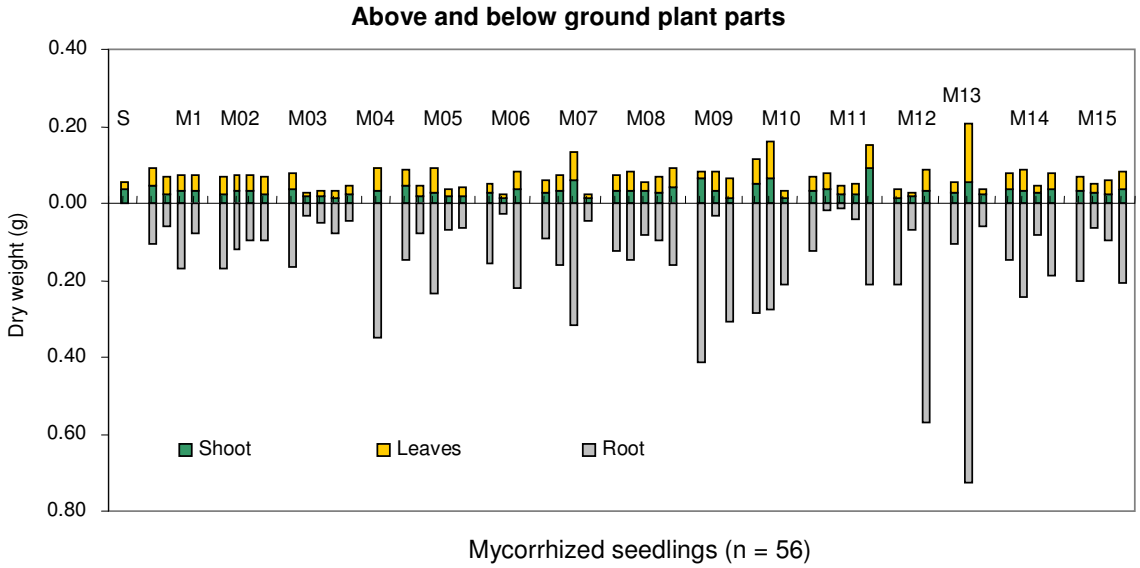


Figure 7b. Shoot and root dry weight (in g per seedling) for individual mycorrhizal (M) (n = 56). S = Seedling before the experiment.

After identifying the non-uniform growth patterns at the end of the experiments, the root weight contribution by individual seedlings were plotted as a histogram for non-mycorrhizal experiment resulting in a normal distribution (Fig. 8a) and for mycorrhizal experiment resulting in a skewed distribution (Fig. 8b).

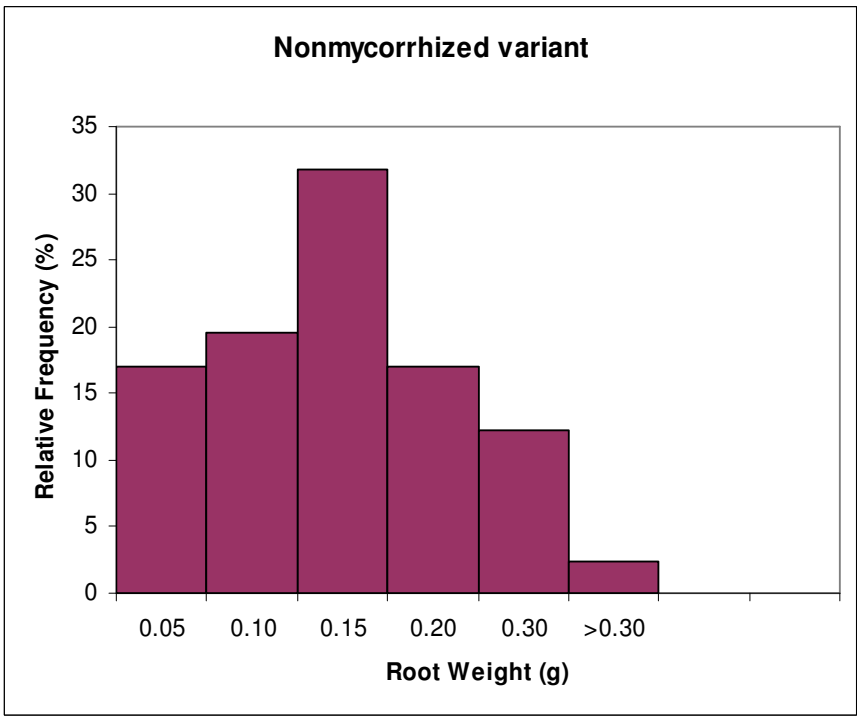


Figure 8a. Frequency distributions of root weights for non-mycorrhizal Scots pine seedlings. (n = 41).

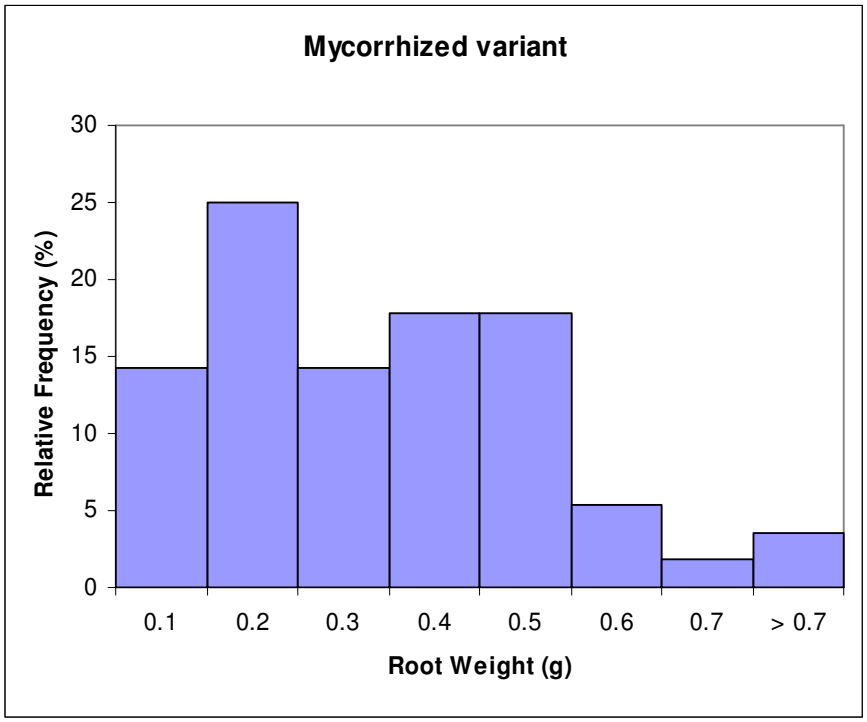


Figure 8b. Frequency distribution of root dry weights for mycorrhizal Scots pine seedlings (n = 56).

This sort of skewness in distribution may be due to the effect of dominance/suppression of the individuals leading to inequal growth with in the individuals. As the individual seedlings of the population contributed disproportionately to the total root weight within each petridish in the experiments, the degree of the dissimilarity is a measure of inequality with in the individuals in a population. This measure of inequality with in the individuals in a population showing the development of relationships of dominance/suppression is known as Gini coefficient ($\overline{G}$). This measure of inequality with in individuals is called as a Lorenz curve of inequality (Fig. 9) and is known by calculating Gini coefficient. When the root weight of the individual plants is similar to each other and if each successive individual is added to the population, the cumulative proportion of the total population weight would increase linearly, resulting in a diagonal line (called the line of equality). When the contribution of root weight by individuals in a population is unequal and if they are ranked from low to high, the bending of curve corresponds to the Gini coefficient. When the area between curve and the linear diagonal line is more, it corresponds to larger Gini coefficient, emphasizing greater inequalities (Bendel et al. 1989).

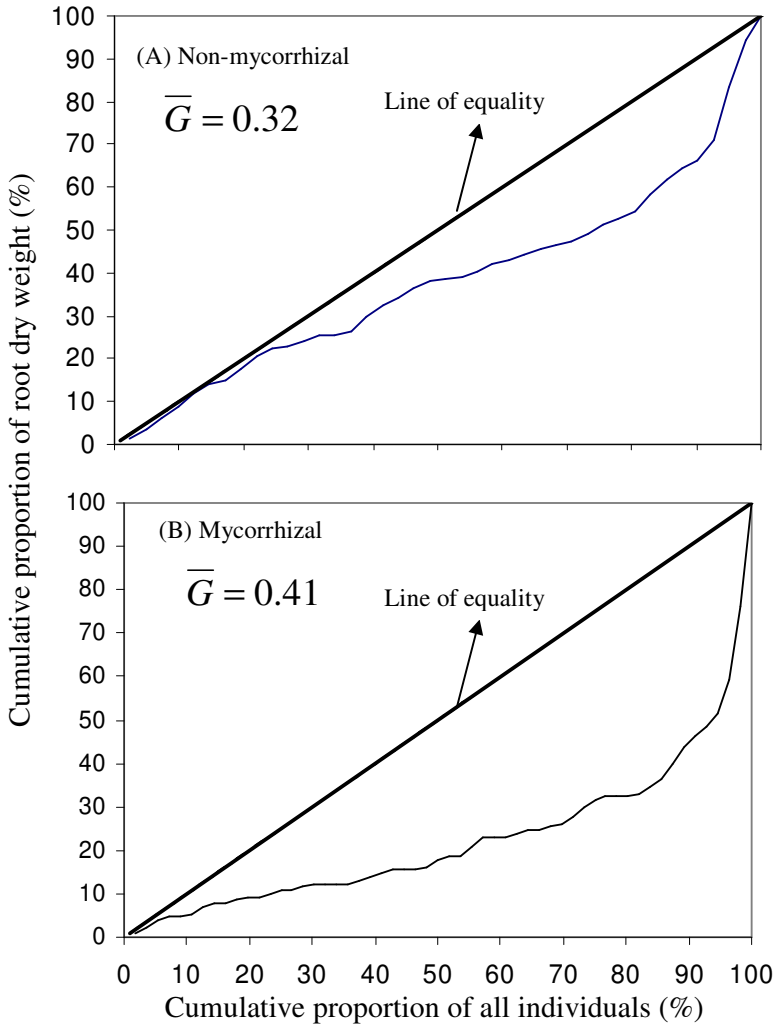


Figure 9. The Lorenz curves for (A) non-mycorrhizal (n = 41) and (B) mycorrhizal seedlings (n = 56) and respective Gini coefficients ($\overline{G}$).

3.2 Distribution of plant biomass in the mycorrhizal and non-mycorrhizal seedlings

The cumulative proportion of the total plant dry mass percentage for both the variants is demonstrated by taking into account, the above ground parts, such as stem and leaves, and below ground part, such as, roots in each petridish (Fig. 10).

The biomass distribution trend with in the individual petridishes of the treatments follows a close pattern to each other with a difference of 10%. The biomass accumulation in mycorrhizal seedlings was lower than the non-mycorrhizal seedlings. However, the hyphae lost due to root washings were not taken into account in calculation of biomass for the mycorrhizal seedlings.

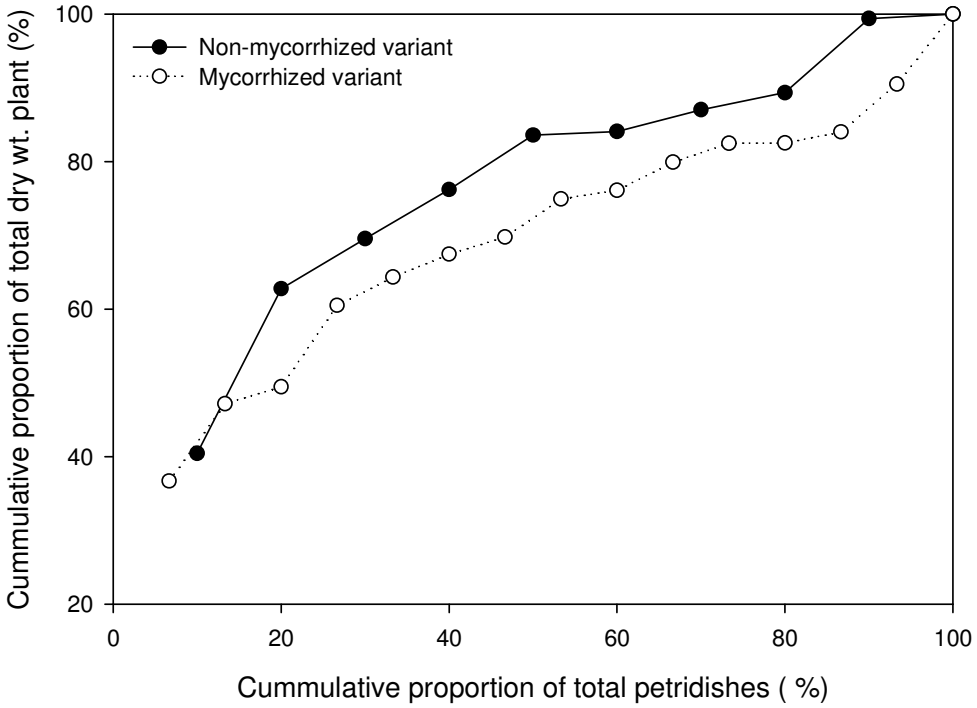


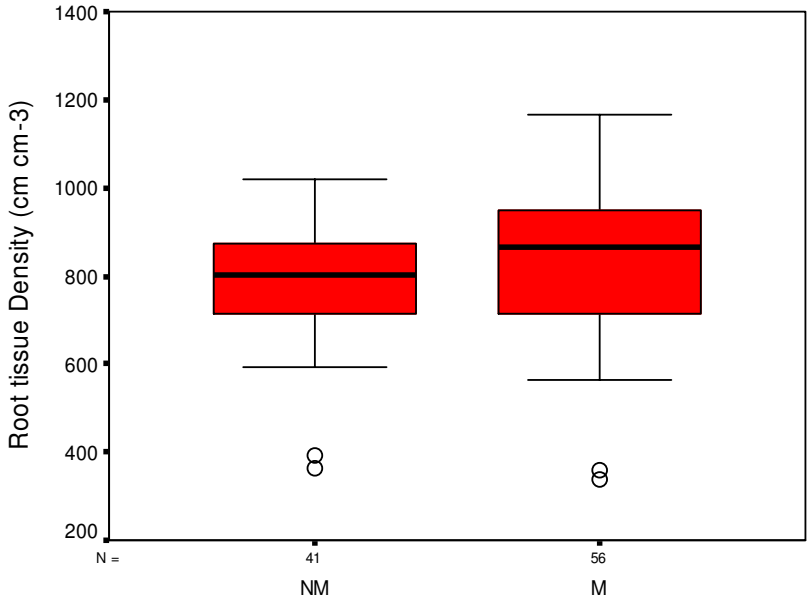
Figure 10. Total biomass distribution in the petridishes of non-mycorrhizal and mycorrhized treatments.

3.3 Plant root tissue density (RTD) and specific root length (SRL) in the mycorrhizal and non-mycorrhizal treatments

Root length and specific area are important indicators for a potential uptake of water and mineral nutrients, and especially root diameter is considered as one of the important input parameters in rhizosphere modelling (Himmelbauer et al. 2004). The plant root tissue density (RTD) is the length of root associated with a unit volume of root tissue (cm cm^{-3}). The spatial distribution of roots is a good indication of abundance of nutrients (Hutchings and John 2003). In the experiments, though the roots were widely distributed

in the mycorrhizal seedlings in comparison to the non-mycorrhizal seedlings, it was not an advantage in terms of nutrient accumulation for mycorrhizal seedlings (Fig. 11).

Specific root length (SRL) is the length of the root per dry weight of the root (cm g^{-1} or m g^{-1}). The function of SRL is the indication of the strategy of roots for allocation of nutrients to the plant by way of better adaptation to the substrate environment and translocation of nutrition. Although, SRL is an indicator of the increase in root length to the given mass of roots, it is more dependent on the type of nutrient, for e.g., Phosphate (Robinson et al. 1999). Using root tissue density together with specific root length, root diameter can be estimated (Atkinson 2000).



NM = Non-mycorrhized variant, M = Mycorrhized variant

Figure 11. Root Tissue Density (RTD, cm cm^{-3}) of non-mycorrhizal and mycorrhizal seedlings ($p > 0.05$). A thick black line within boxes represents the median, separating above and below percentile boundaries, and the bars extend to the maximum and minimum values.

Specific root length (SRL) was similar in both the mycorrhizal and non-mycorrhizal seedlings, with only two extreme values on higher side in mycorrhizal experiment and showed no significant variation between the treatments with respect to adaptation to the living conditions (Fig. 12).

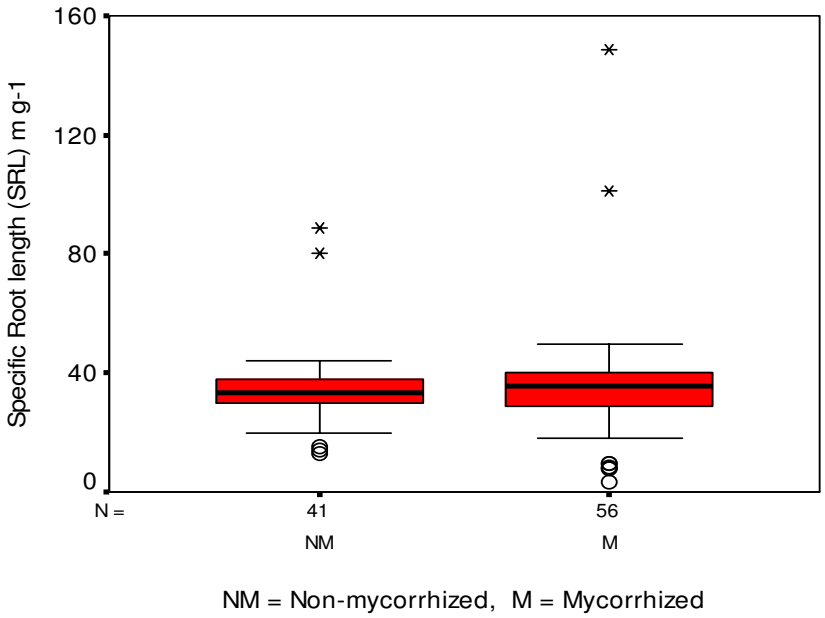


Figure 12. Specific root length (SRL, m g^{-1}) of non-mycorrhizal and mycorrhizal seedlings ($p > 0.05$). A thick black line within boxes represents the median, separating above and below percentile boundaries, and the bars extend to the maximum and minimum values.

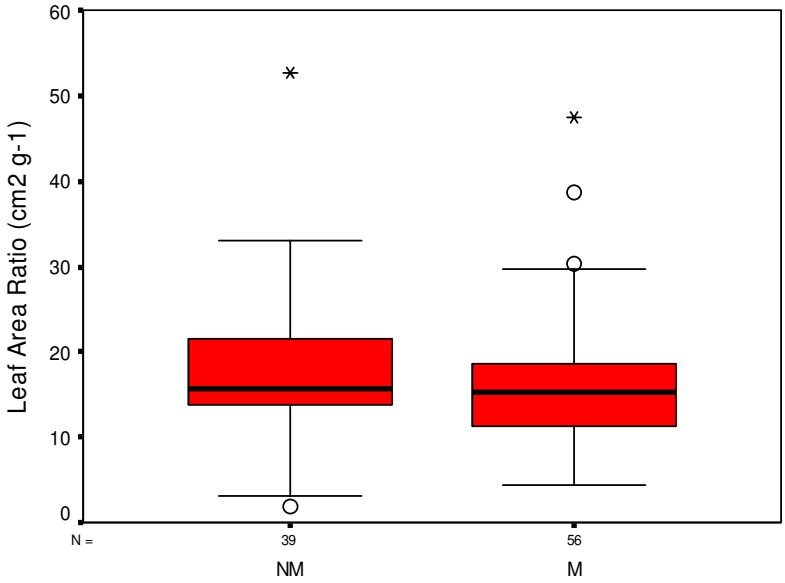
3.4 Plant Leaf Area Ratio (LAR) distribution in the mycorrhizal and non-mycorrhizal treatments

Leaf area ratio (LAR or **F)** is a complex function determining the effectiveness of the photosynthesis, i.e., dependence on light. It gives information of proportion of plant weight devoted to the leaf material (Fitter and Ashmore 1974).

As the leaf area ratio (F) is derived from leaf weight ratio (LWR) (g g^{-1}) multiplied by specific leaf area (SLA) (cm^2 g^{-1}), the units for LAR are expressed as cm^2 g^{-1}.

Where, LWR is the ratio of leaf weight to the total plant weight and SLA is the ratio of leaf area to the photosynthetically active leaf weight.

The leaf area ratio (LAR) shows similar leaf functionalities in both the non-mycorrhizal and mycorrhizal seedlings, indicating having similar photosynthetic abilities (Fig. 13).



NM = Non-mycorrhized variant, M = Mycorrhized variant

Figure 13. Leaf Area Ratio (LAR) of non-mycorrhizal and mycorrhizal seedlings ($p > 0.05$). A thick black line within boxes represents the median, separating above and below percentile boundaries, and the bars extend to the maximum and minimum values.

3.5 C, N and S allocation in Scots pine seedlings

Regarding the non-uniform contribution of biomass of individual seedlings in the non-mycorrhizal and mycorrhizal treatments (section 3.2), only the robust and dominant plants were selected for the analysis of C, N, and S contents as the highest biomass contributor in each petridish. The purpose of choosing maximum deterministic individual seedling was to reduce the samples in each petridish for analysis of C, N and S. In the experiments only the roots and needles of the selected robust plants were analyzed.

3.5.1 C, N and S contents in root dry mass of the non-mycorrhizal and mycorrhizal pine seedlings

Analyzed C, N and S, percentages data of the root mass derived from CNS analyzer were re-calculated by applying excess quartz sand weight corrections which is known by weighing remaining quartz particles from acid (65% HNO_3) digestion and ashing of filter paper (as described in section 2.8.4). The quartz sand corrections were applied using the correction factors (appendix Tab. A5a and A5b). The mean C : N ratio in non-mycorrhizal (Tab. 5a) was higher in comparison to the mycorrhizal seedlings (Tab. 5b), possibly due to carbon supply to the mycorrhizal symbiont.

Table 5a. Recalculated C-, N- and S- concentrations (%) in dry root mass per petridish (n =10) of non-mycorrhizal seedlings after applying root weight corrections. Mean values are denoted by $\overline{X}$.

Serial Number of petridish	After root weight corrections			
	C %	N %	S %	C:N
N1	35.19	0.65	0.15	56.8
N2	31.05	0.60	0.30	54.5
N3	41.97	0.73	0.25	51.8
N4	44.27	0.72	0.19	57.8
N5	43.31	0.59	---a)	61.5
N6	41.19	0.58	---a)	72.9
N7	36.21	0.52	---a)	70.8
N8	40.26	0.58	---a)	69.5
N9	39.64	0.67	---a)	69.3
N10	42.75	0.61	---a)	59.2
	$\overline{X} = 39.59$	$\overline{X} = 0.63$		$\overline{X} = 63.7$

---[a)] Recalculation for values <0.1 was not possible

Table 5b. Recalculated C-, N- and S- concentration (%) in dry root mass per petridish (n = 15) of mycorrhizal seedlings after applying root weight corrections. Mean values are denoted as $\overline{X}$.

Serial Number of petridish	After root weight corrections			
	C %	N %	S %	C:N
M1	41.71	0.78	0.30	53.2
M2	38.08	0.66	0.26	57.9
M3	38.14	0.57	0.24	66.9
M4	35.49	0.59	0.26	59.9
M5	39.53	0.65	0.25	61.0
M6	39.53	0.69	---a)	57.6
M7	40.22	0.68	---a)	59.3
M8	35.48	0.66	---a)	54.0
M9	35.26	0.60	0.23	58.5
M10	44.26	0.80	0.25	55.2
M11	41.53	0.86	0.30	48.4
M12	na	na	na	na
M13	27.41	0.40	---a)	69.4
M14	32.13	0.61	---a)	52.4
M15	37.56	0.66	---a)	53.2
	$\overline{X} = 37.6$	$\overline{X} = 0.63$		$\overline{X} = 57.1$

---[a)] Recalculation for values <0.1 was not possible;
na- values not available.

3.5.2 C, N and S contents in needle dry mass of the non-mycorrhizal and mycorrhizal pine seedlings

The mean C : N ratio in the needles of mycorrhizal seedlings (Tab. 6a) was slightly higher in comparison to the non-mycorrhizal seedlings (Tab. 6b), possibly due to higher photosynthetic activity to support the mycorrhizal symbiont.

Table.6a. Analyzed C-, N- and S- concentrations (%) of needles for non-mycorrhizal seedlings (n = 10) with mean value ($\overline{X}$).

Serial Number of petridish	C %	N %	S %	C:N
N1	45.10	1.11	0.11	40.6
N2	46.77	0.98	0.18	48.0
N3	45.75	1.16	< 0,1	39.6
N4	45.65	1.01	0.13	45.2
N5	46.25	0.85	0.17	54.4
N6	45.84	0.91	< 0,1	50.4
N7	44.75	0.93	< 0,1	48.4
N8	45.40	0.92	< 0,1	49.6
N9	46.77	1.16	< 0,1	40.5
N10	45.49	0.85	< 0,1	53.5
				$\overline{X} = 47.0$

Table 6b. Analyzed C-, N- and S- concentrations (%) of needles for mycorrhizal seedlings. (n = 15) with mean value ($\overline{X}$).

Serial Number of petridish	C %	N %	S %	C:N
M1	47.18	1.15	0.13	41.2
M2	48.84	0.89	< 0,1	54.9
M3	48.05	0.97	< 0,1	49.8
M4	48.13	1.28	< 0,1	37.6
M5	48.51	0.86	0.18	56.4
M6	47.54	1.28	0.20	37.3
M7	46.80	1.13	0.14	41.6
M8	47.58	0.78	< 0,1	61.4
M9	46.94	0.69	< 0,1	68.5
M10	47.49	1.06	< 0,1	45.0
M11	46.68	0.84	0.15	55.6
M12	48.67	1.10	0.12	44.2
M13	48.40	0.75	0.13	64.5
M14	46.15	0.89	0.24	51.9
M15	47.97	0.96	0.20	41.2
				$\overline{X} = 50.7$

3.6 Element distribution in shoots and roots of the non-mycorrhizal and mycorrhizal seedlings

After the 8 months growth, the seedlings were separated into stem, needles and roots. The elements analyzed were phosphorus (P), calcium (Ca), potassium (K), magnesium (Mg), iron (Fe) and manganese (Mn) in non-mycorrhized seedlings. For comparative purposes, the nutrient element present at the beginning of the experiments S (n = 5) are also shown to mark the common starting point. Before the start of the experiments the phosphorus content was higher in the germinated seedling and after the growth, equal distribution was observed in the above and below ground parts. For the elements, such as, calcium, potassium and magnesium, distribution into above and below ground parts was equal for individual seedlings. More accumulation of iron was in the roots than the stem and the needles together. Conversely, more accumulation of manganese was in the above ground parts than in the roots of the non-mycorrhizal seedlings (Fig. 14a).

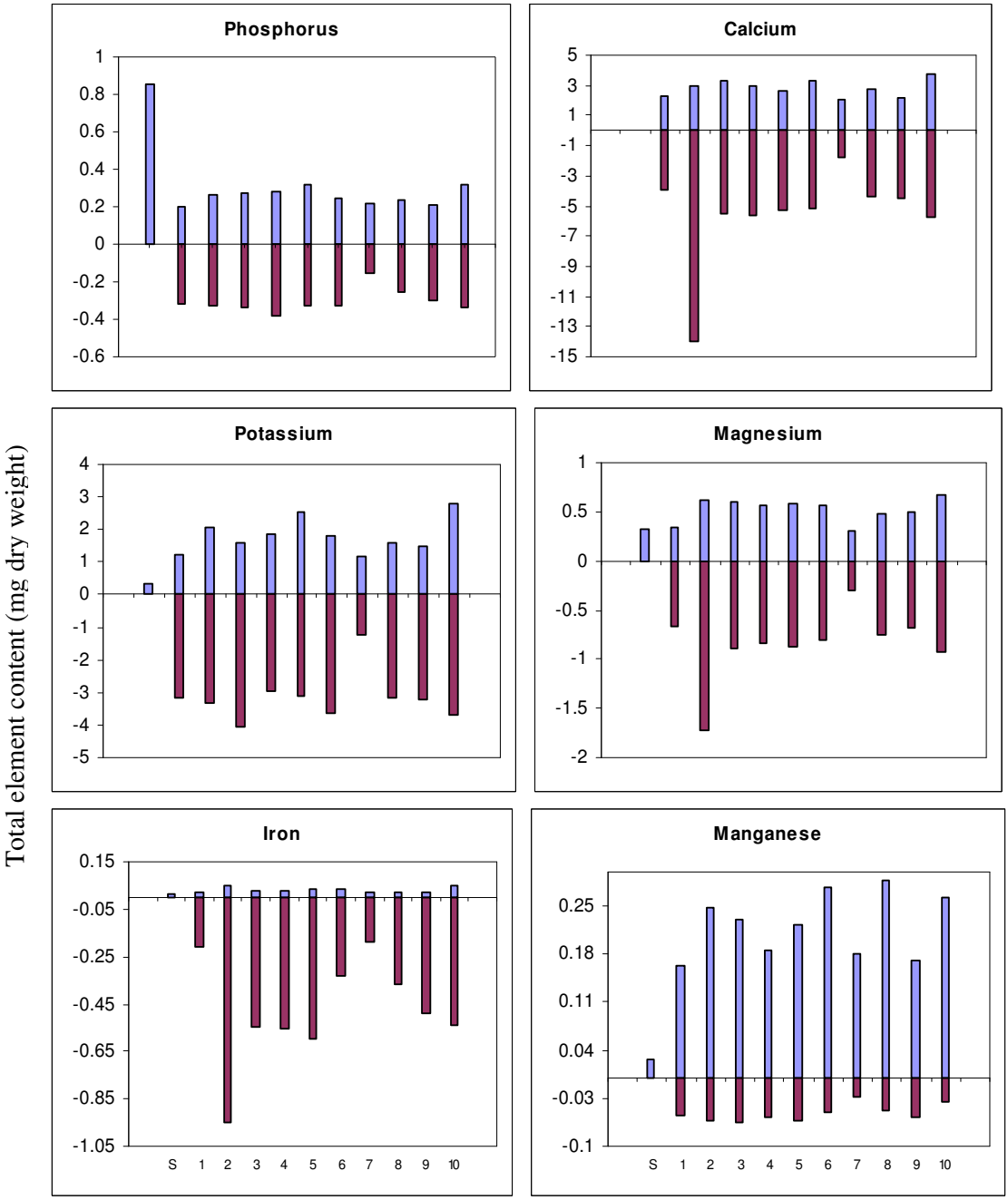


Figure 14a Total element content in non-mycorrhizal seedlings in petridishes (N = 10). Nutrient status at the beginning of the experiments, 'S' (n = 5). Positive values are for 'above ground' and negative values are for 'below ground' parts.

Similar to the non-mycorrhizal experiment, the seedlings in the mycorrhizal seedlings were analyzed for phosphorus (P), calcium (Ca), potassium (K), magnesium (Mg), iron (Fe) and manganese (Mn) elements. For comparative purposes the elements present at the beginning of the experiments S (n=5) are also shown to mark the common starting point.

Before the start of the experiments the phosphorus content was higher in the germinated seedling and after the growth, more phosphorus is observed in the roots than the stem and needles together. Similarly, the elements such as, calcium, potassium and magnesium were distributed more in the roots than the stem and needles together in the individual seedlings. More accumulation of iron was in the roots than the above ground parts. Conversely, more accumulation of manganese was in the above ground parts than in the roots of the mycorrhizal seedlings (Fig. 14b).

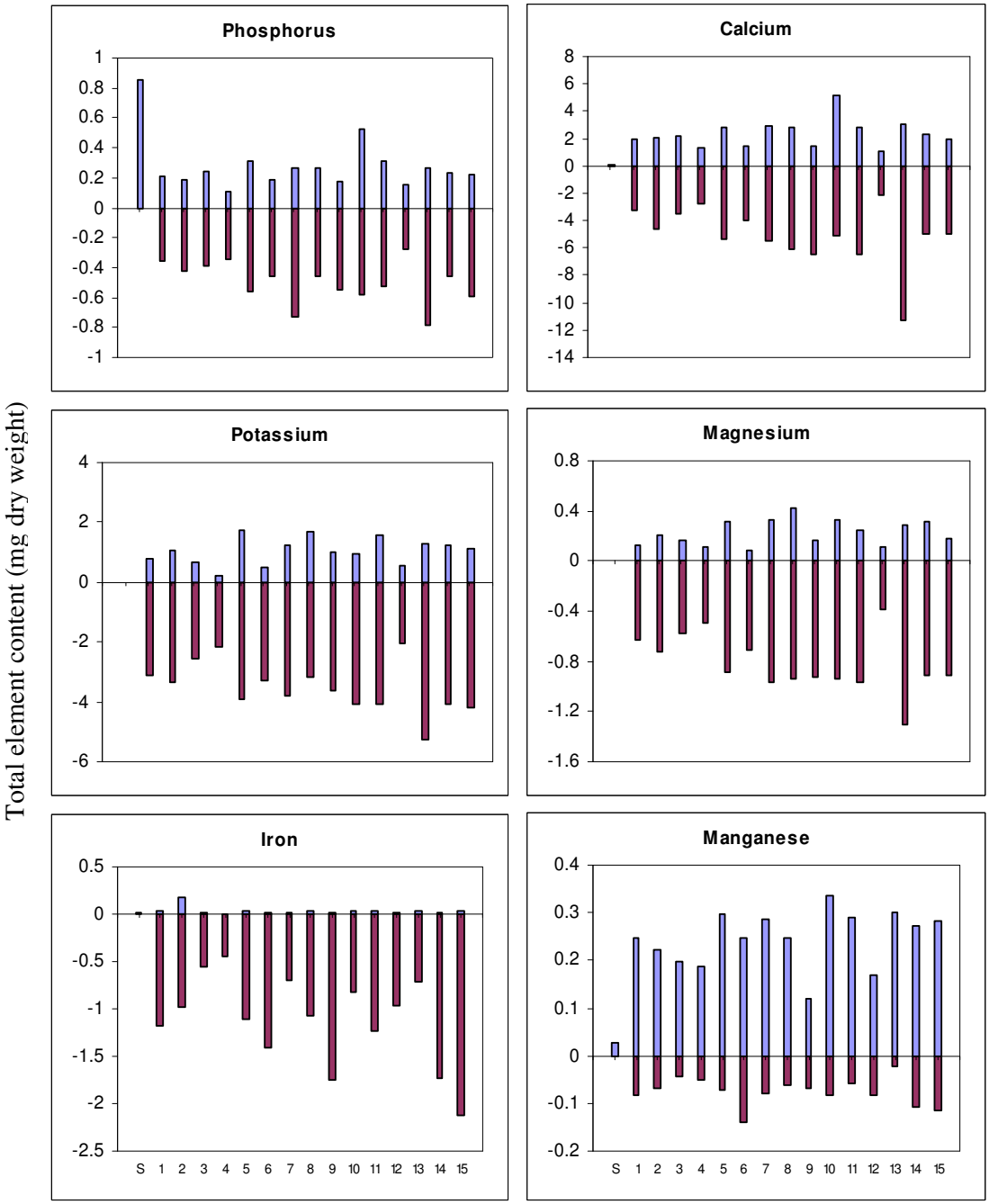


Figure 14b Total element content in non-mycorrhizal seedlings in petridishes (N = 15). Nutrient status in the seedling at the start of the experiments, 'S' (n = 5). Positive values are for 'above ground' and negative values are for 'below ground' parts.

In both the non-mycorrhizal and mycorrhizal seedlings (shoot and roots), the nutrient element accumulation of calcium, potassium, magnesium were similar ($p > 0.05$). Higher iron and phosphorus accumulation ($p < 0.05$) was in mycorrhizal seedlings in comparison to the non-mycorrhizal seedlings, followed by manganese element ($p = 0.063$) in the mycorrhizal seedlings, which was taken up in slightly higher quantity than the non-mycorrhizal seedlings (Fig. 15).

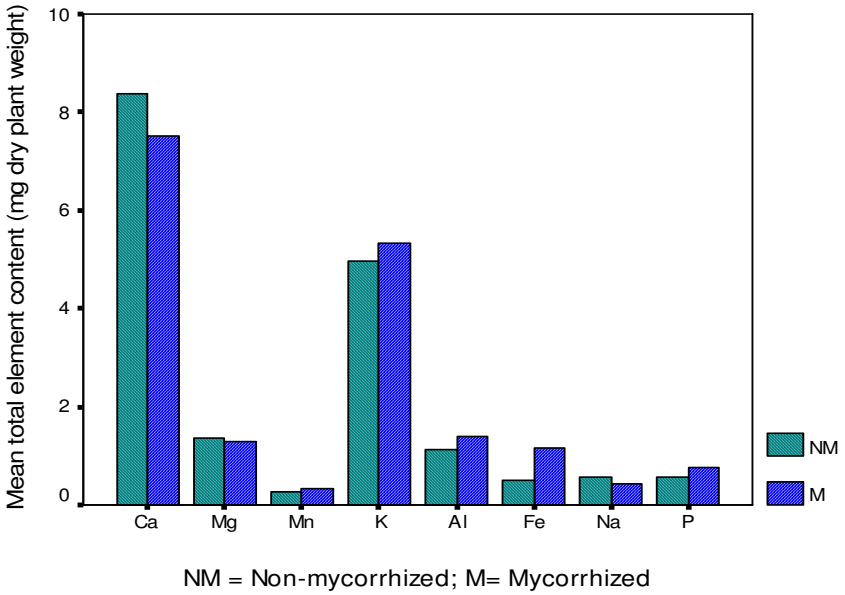


Figure 15. Mean total element contents in Non-mycorrhizal (NM) and Mycorrhizal (M) seedlings (shoot and roots).
Ca, Mg, Al, Na, K (not significant); Mn ($p = 0.063$); Fe, P ($p < 0.05$).

3.7 pH and cation concentration in the rhizosphere soil solutions

3.7.1 pH in the rhizosphere soil solutions

The rhizosphere solutions were measured for pH. Only the soil solutions sampled at the end of the experiments were sufficient to measure pH after analyzing for LMW carboxylates and cations to avoiding interference with the analysis. The pH values of solutions taken from the rhizosphere solutions of mycorrhizal and non-mycorrhizal seedlings were not significantly different. However, it is noteworthy that the 50 percentile of pH values of the mycorrhizal experiments fall in the range between 5.5 to 4.5 and 75 percentile of pH values of non-mycorrhizal experiments are ranging between 5.5 to 6.5 (Fig. 16) leading to more cation availability for uptake in the mycorrhizal seedlings.

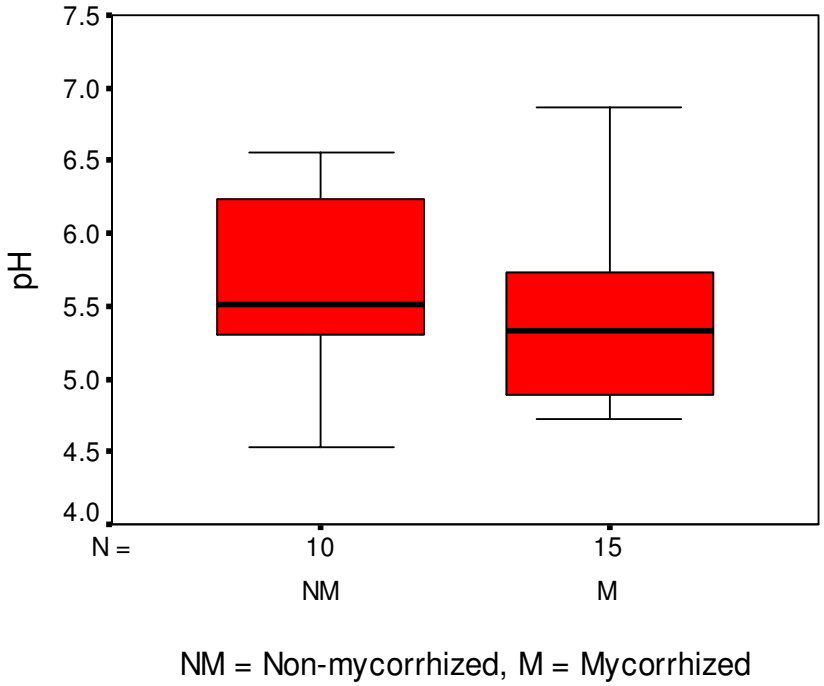


Figure 16. pH in the rhizosphere solutions of non-mycorrhizal and mycorrhizal experiments. A thick black line within boxes represents the median, separating above and below percentile boundaries, and the bars extend to the maximum and minimum values.

3.7.2 Cation concentration in the rhizosphere solutions of non-mycorrhizal and mycorrhizal pine seedlings

At the end of the experiment, the nutrient supply was stopped and only distilled water supplement was provided for 3 weeks. A final sample of the rhizosphere soil solution was sampled to comparatively analyze the labile fraction of cations, such as, K^+, Na^+, Ca^{2+}, Mg^{2+} and $Fe^{2+/3+}$ by means of capillary electrophoresis. Considerable amount of cations are present in the rhizosphere in ionic form and, thus, are available for uptake. Concentrations of labile cations are always higher in rhizosphere solution of mycorrhizal seedlings than in non-mycorrhizal seedlings. As to pH > 4.0 in the rhizosphere solutions, it was not possible to detect the concentrations of Al^{3+} and Mn^{2+} by CE analysis (Fig.17).

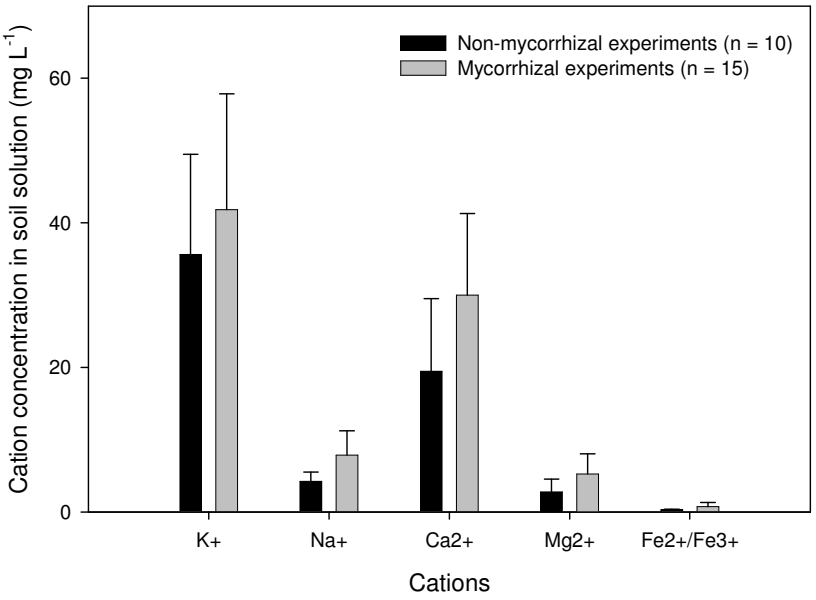


Figure 17. Labile cations in rhizosphere solutions of non-mycorrhizal and mycorrhizal treatments analyzed by CE (bars indicate ± SD).

3.8 Comparative analysis of Low Molecular Weight carboxylate concentration in the rhizosphere solutions by High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE)

The exudates (LMW carboxylates) (Fig. 18) in the rhizosphere soil solutions were analyzed using two different instruments. The exudates were analyzed by using HPLC for the carboxylic acids (R-COOH) forms such as, oxalic, L-malic, citric, lactic and succinic acids. The exudates were analyzed by using CE for the enolate ($-COO^-$) forms, such as, oxalate, citrate, L-malate and succinate. Lactate was not analyzed due to poor detection or no detection depending on the buffer used in comparison to the other carboxylates on CE. The standard solutions were prepared in laboratory (same standards used for analysis by CE and HPLC) and are were in concentrations of 1 mg L^{-1}, 2 mg L^{-1}, 3 mg L^{-1}, 4 mg L^{-1}, 5 mg L^{-1} and 7 mg L^{-1}. The peak signals of the standard curves for the standard solutions were tabulated and the calibration curve equations were used to calculate the concentrations of the carboxylates in the rhizosphere solution samples (appendix Tab. A4a and Tab. A4b).

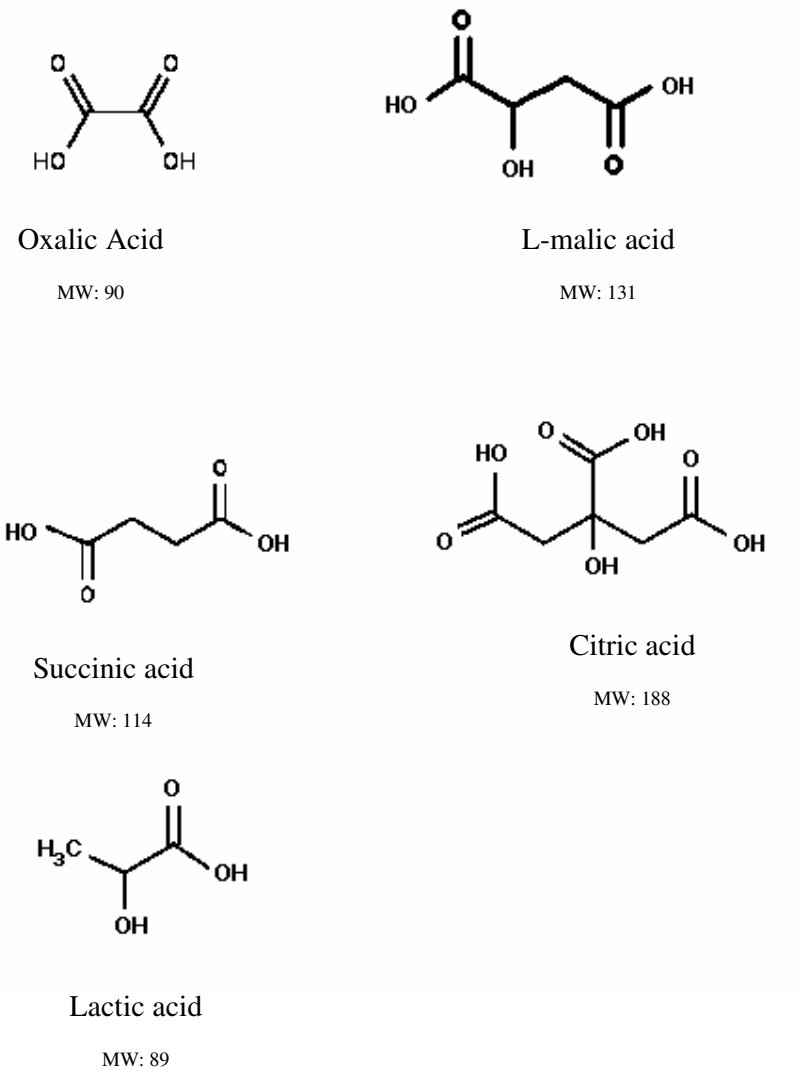


Figure 18. LMW carboxylates detected in the rhizosphere solutions.

Of the exudates sampled between the 3rd month and 8th month of the experiments, it is noteworthy that oxalate concentration was higher than other LMW carboxylates in the mycorrhizal than non-mycorrhizal seedlings. In comparison to oxalate, other carboxylates were in low concentrations (Fig. 19). As the oxalic acid peak was co-eluting with the injection peak during the HPLC analysis, oxalic acid concentration was discarded and only the citric, L-malic and lactic acids were accepted. Hence, from the HPLC/CE analysis of exudates data, only oxalate/oxalic acid was considered as the main influencing factor in the rhizosphere soil solutions. Thus, oxalate analyzed by using CE being the dominant analyte was focused mainly in this thesis work.

The oxalate concentrations in non-mycorrhizal experiments were fluctuating over the sampling period and stabilized only towards the end of the 8th month. For the mycorrhizal

experiments variation in oxalate concentrations were fairly stable over the sample periods (Fig. 20). The oxalate concentrations were produced in significantly higher amounts (p = 0.01) in the rhizosphere soil solution of the mycorrhizal than the non-mycorrhizal experiments (Fig. 21).

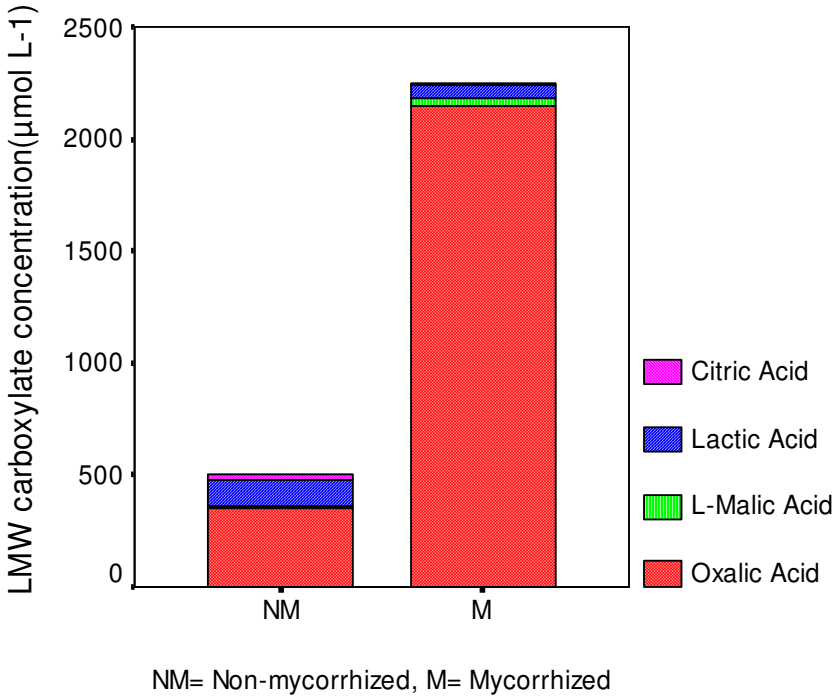


Figure 19. Influence of mycorrhization on the release of exudates into the rhizosphere solutions of Scots pine seedlings (analyzed on HPLC).
Oxalic acid (p=0.02); L-malic acid (p=0.05); citric acid & lactic acid (not significant).
*Citric acid and *lactic acid are found in large concentrations mostly in Non-mycorrhized variant.

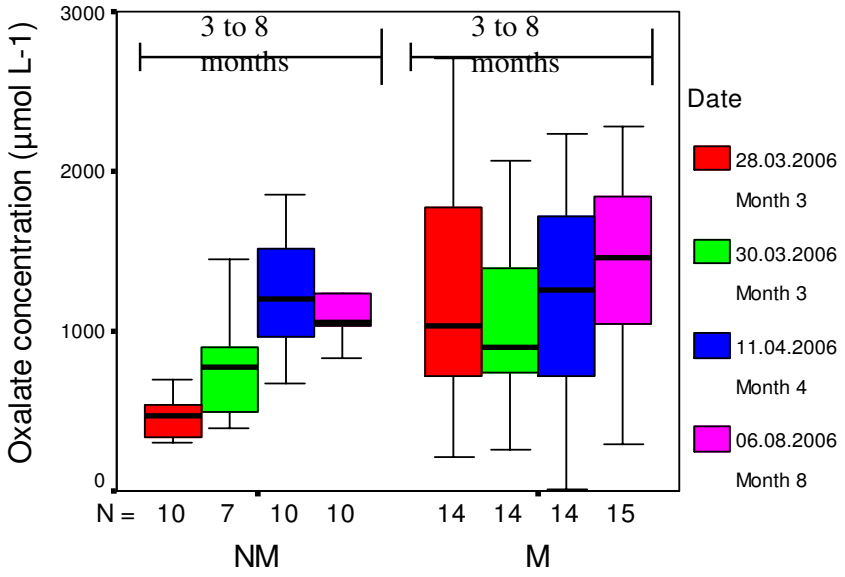


Figure 20. Oxalate concentrations in the rhizosphere solution of non-mycorrhizal and mycorrhizal treatments. A thick black line within boxes represents the median, separating above and below percentile boundaries, and the bars extend to the maximum and minimum values.

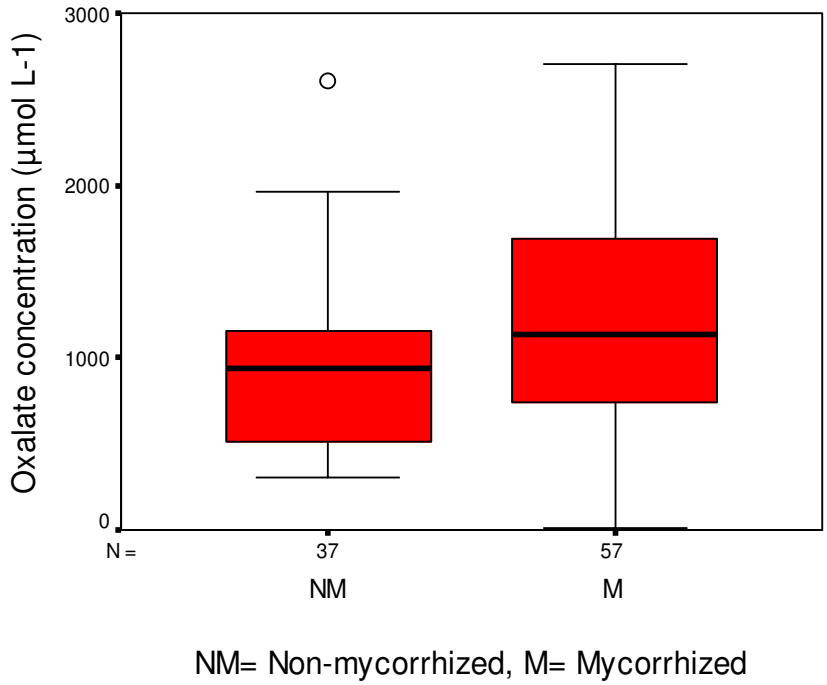


Figure 21. Influence of mycorrhization on the release of oxalate in the rhizosphere solutions of Scots pine seedlings as being detected by CE ($p = 0.01$). A thick black line within boxes represents the median, separating above and below percentile boundaries, and the bars extend to the maximum and minimum values.

3.9 Analysis of influence of Mycorrhization depending on experimental parameters

Principle Component Analysis (PCA) was used to analyze the effect of mycorrhization in a statistically representative way. PCA is suitable tool to study the variability of morphological and chemical parameters. First principle component (PC1) accounts for 66.9% and second principle component (PC 2) for 19.6% of the data clearly differentiating between mycorrhizal and non-mycorrhizal seedlings. The outcome of PCA was limited to just these axes when describing the variation. Depending on the influence of mycorrhization of Scots pine (*Pinus sylvestris*), a demarcated zone of mycorrhizal and non-mycorrhizal experiments is plotted (Fig. 22), clearly showing that the state of mycorrhization has a significance influence on the chemical composition of the rhizosphere solution.

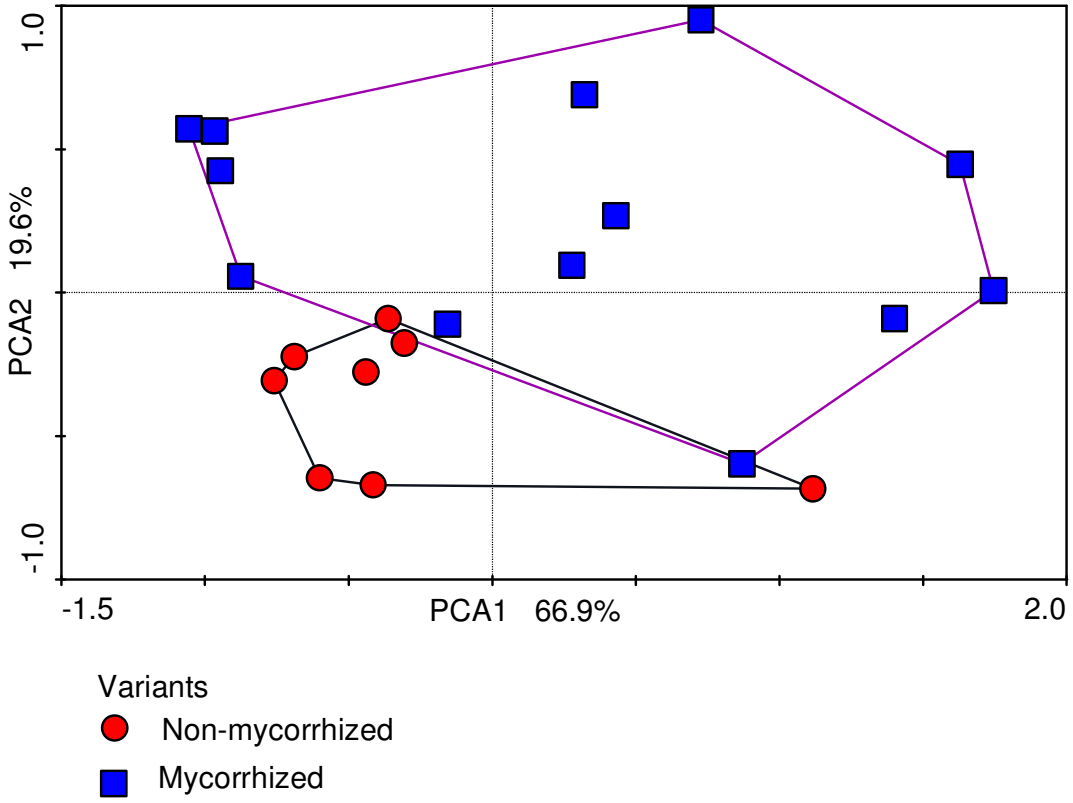


Figure 22. Principle component analysis with first principle component (PCA1) describing 66.9% and second principle component (PCA2) describing 19.6% of overall variation due to mycorrhization. The non-mycorrhizal experiments are represented by circles and mycorrhizal experiments by squares.

Further, to explore how the different types of LMW carboxylates relate to the presence and absence of mycorrhization, principle component analysis was carried out (Fig. 23).

The arrows represent the LMW carboxylates, such as, oxalate, citric acid, lactic acid and L-malic acid, analyzed in the rhizosphere soil solutions of the treatments. The experiment points lying in the direction of the arrow correlate to higher concentrations of LMW carboxylates. Correspondingly, if the arrows are extended to the opposite side (backwards) from the origin in the centre; the points lying along this virtual arrow imply having lower or even negligible concentrations of the LMW carboxylates in the rhizosphere soil solutions. However, for the non-mycorrhizal seedlings, exudates other than oxalate appear more frequently, indicating that the absence of the symbiotic fungus is compensated for a release of more specific organic acids that otherwise were not needed for nutrient acquisition.

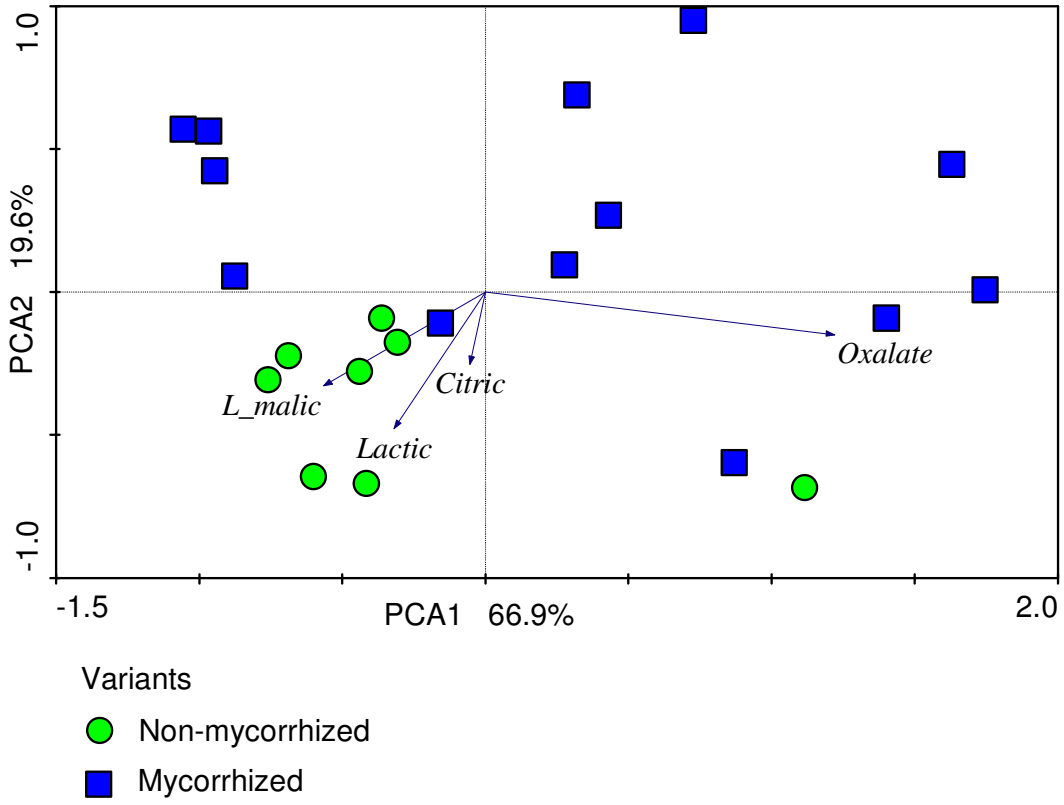


Figure 23. Correlations of types of LMW carboxylates measured in the experiments. The non-mycorrhizal experiments are represented by circles and mycorrhized experiments by squares.

The fact that oxalate was clearly dominating in the spectrum of LMW carboxylates and differentiating between mycorrhizal and non-mycorrhizal seedlings is illustrated with the oxalate as the determining parameter (Fig. 24). The oxalate concentrations are shown in the form of a diamonds, in varying sizes in accordance to low to high concentrations analyzed in the rhizosphere soil solutions. The non-mycorrhizal experiments are represented by green coloured diamonds and mycorrhizal experiments by blue coloured diamonds.

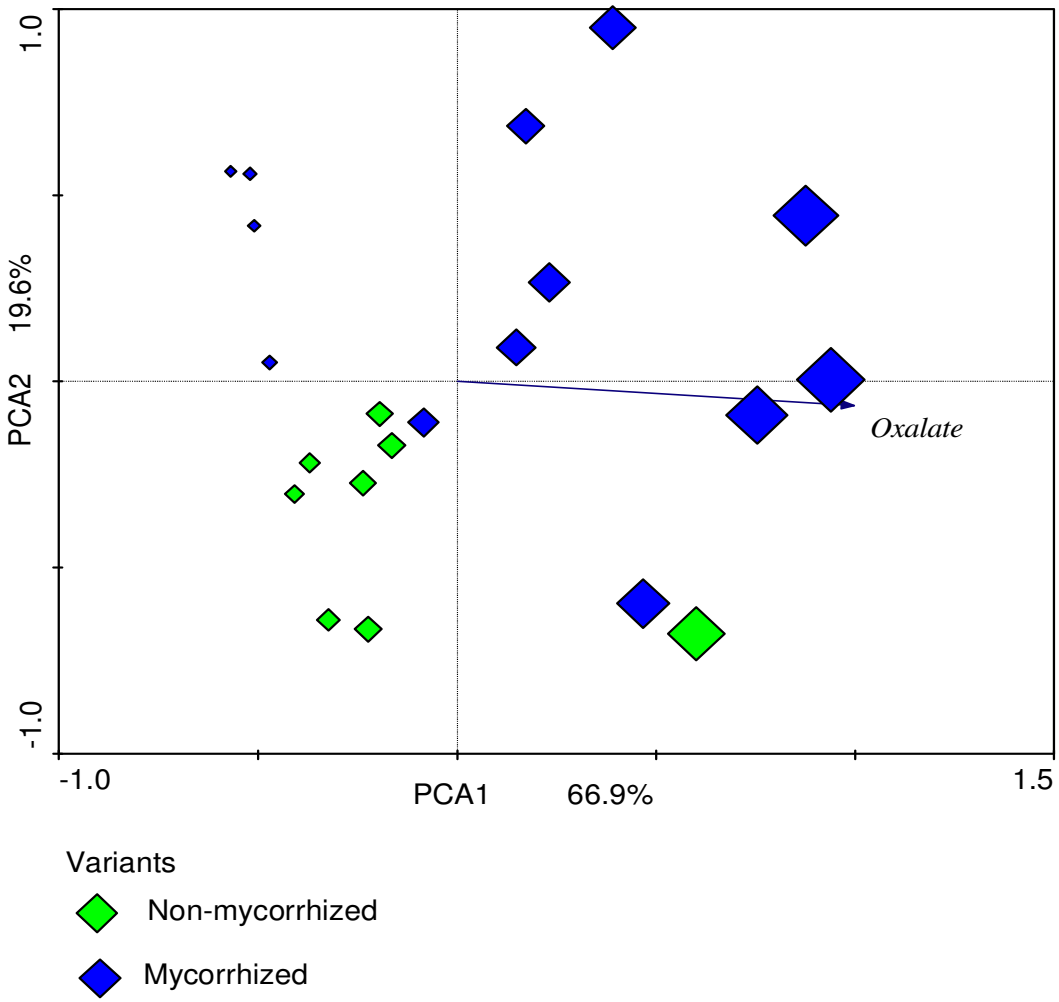


Figure 24. Principle component analysis chart illustrating oxalate at different concentrations in the mycorrhizal (blue coloured diamonds) and in non-mycorrhizal experiments (green coloured diamonds) with the direction of arrow pointing towards to higher concentrations.

Another PCA was applied to test the influence of anions (oxalate and nitrates) on variation of pH in the rhizosphere solutions of mycorrhizal and non-mycorrhizal experiments. The concentrations of oxalate, nitrate and the pH values measured in the

rhizosphere soil solutions of both experiments are represented as arrows. As the oxalate concentrations were found to be higher in the mycorrhized experiments, the pH value is found to be lower, i.e., towards acidic. Correspondingly, low concentrations of oxalate and high concentrations of nitrate in non-mycorrhized correlates with higher pH, i.e., towards alkaline (Fig. 25).

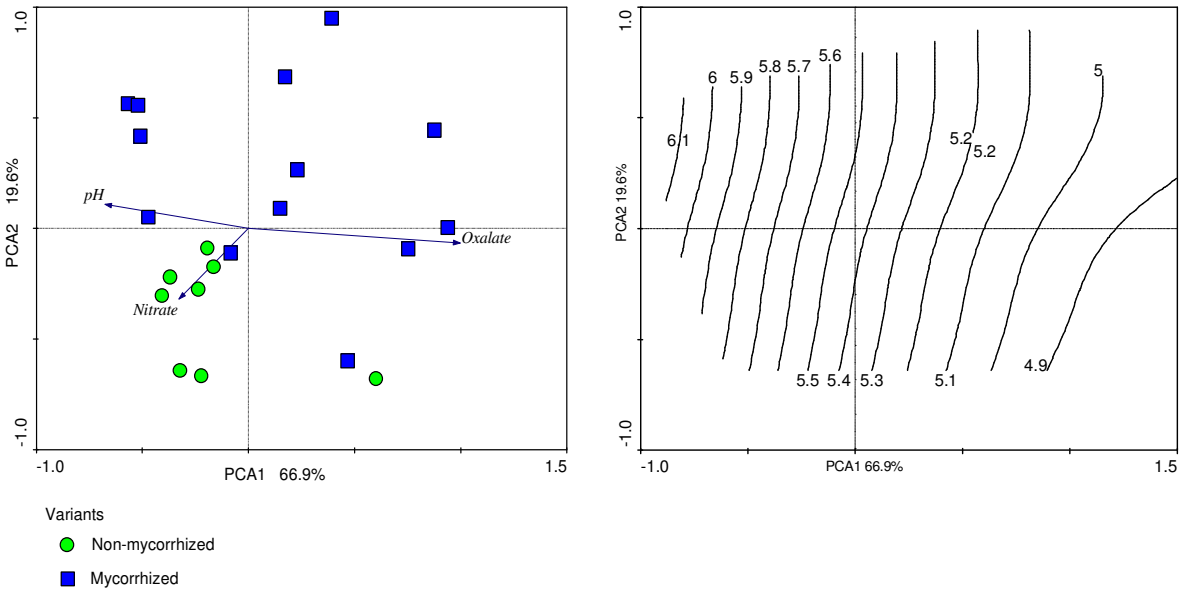


Figure 25. Principle component analysis reflecting influence of anions (oxalate and nitrate, represented as arrows) as pH regulators in the rhizosphere soil solutions and the pH attributed (contours) by the specimens of non-mycorrhizal (circles) and mycorrhizal experiments (squares).

3.10 Comparison of base cation contents in rhizosphere soil solution and in the plant tissue for mycorrhizal and non-mycorrhizal treatments in mycorrhizal and non-mycorrhizal treatments

Based on the LMW carboxylates analyzed it was hypothesized that at a given point of time, the LMW carboxylate-cation complex may be one of the probable path ways of cation availability as nutrient in the substrate for the plant uptake. The outcome of this hypothesis is a conceptual model.

The estimation of possible organo-metallic complexes was based on the cations in the nutrient solution supplied and the analyzed LMW carboxylates, such as, oxalate, malate and citrate, as the complexing agents found in the rhizosphere soil solutions of the non-mycorrhized and mycorrhized experiments. Lactic acid is not considered for complexation as it has very low ability to form organo-metallic complexes (Jones 1998).

The equation below explains the formation of organo-metallic complexes:

$$M^{n+} + H_nX^{n-} \xleftrightarrow{K1} MH_nX^{-} \text{ or } MX^{-} \quad \ldots\ldots\ldots 5$$

where, M^{n+} is cations (Ca^{2+}, Mg^{2+}…), H_nX^{n-} is the LMW carboxylate and MH_nX^{-} or MX^{-} is the organo-metallic complex. This organo-metallic complex is the precursor for the organically complexed cation estimation.

To calculate the possible organic (LMW acids) + cation complexes in the rhizosphere soil solutions on the basis of above mentioned equation 5, PHREEQC - a geochemical modelling tool was used.

The cations concentrations were calculated from the Hoagland nutrient (Appendix Tab. A1) solution supplied to the petridishes with the two variants. The pH was set to the measured pH in each petridish of the variant.

An example of the input file is given below.

K	234.75 mg L^{-1}
Ca	189.05 mg L^{-1}
Mg	43.29 mg L^{-1}
Na	4.15 mg L^{-1}
Mn	0.50 mg L^{-1}
Al	4.38 x 10^{-03} mg L^{-1}

The LMW carboxylates taken as input variable were, for e.g.,

Oxalate	9.6	mg L^{-1}
Malate	1.36	mg L^{-1}
Citrate	1.05	mg L^{-1}

Hence, using the equations given in the database PHREEQC calculates the complexes depending on the stability constants. For e.g., to calculate the oxalate-Al complex, it is given as

Al^{3+} + oxalate^{2-} = Al(oxalate)$^{+}$ LogK 6.53

From the output file containing the molality for the organometallic complex (Appendix 6), free cation (for eg., Al^{3+}) concentration was known from the molar ratios as mg kg^{-1}.

Depending on the cations calculated as LMW carboxylate-cation complexes (Appendix 6; M1 to M15), comparisons between the plant accumulated cation to the corresponding element in the rhizosphere soil solutions were developed as conceptual models for the mycorrhizal experiments (Fig. 26 to 29). The plant accumulated element, expressed as mg kg^{-1}, was plotted on y-axis in the charts and the element, derived as carboxylate-complex, was plotted on x-axis, from low to high concentrations. The horizontal regression line is the calculated mean for plant accumulated element concentration. For comparison of the LMW carboxylate complexed cation concentration

(mg kg^{-1}) to the accumulated cation concentration in the whole plant, the measured element content in the dry plant mass was transformed into concentration on the basis of biomass of the seedling. Thus, both the concentration axes in the plot are in mg kg^{-1}.

The comparison of plant element concentration to the availability of cation in the substrate describes the influence of mycorrhizal fungal treatment on the exudation by seedlings. In the conceptual models, the highest element concentrations in the rhizosphere are compared to the mean element concentrations in plant. In the mycorrhized experiments the availability of Ca, Mg and K in the rhizosphere soil solutions was more than the mean element concentrations in the plant (Fig. 26; 27 and 28), suggesting high availability of these elements. With regards to Mn, the highest concentrations in the rhizosphere solutions were barely same to the plant Mn concentrations, suggesting low availability of Mn element in the rhizosphere solutions (Fig. 29).

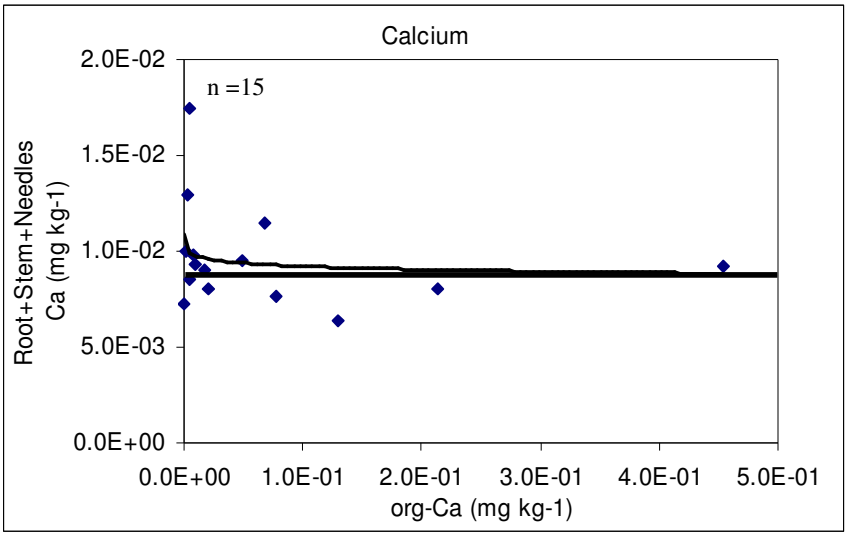


Figure 26. Calcium concentration in whole plant (plant-Ca) to the calcium availability as LMW carboxylate complex (org-Ca) in mycorrhizal experiment (n =15). (Mean = $9.0E^{-03}$).

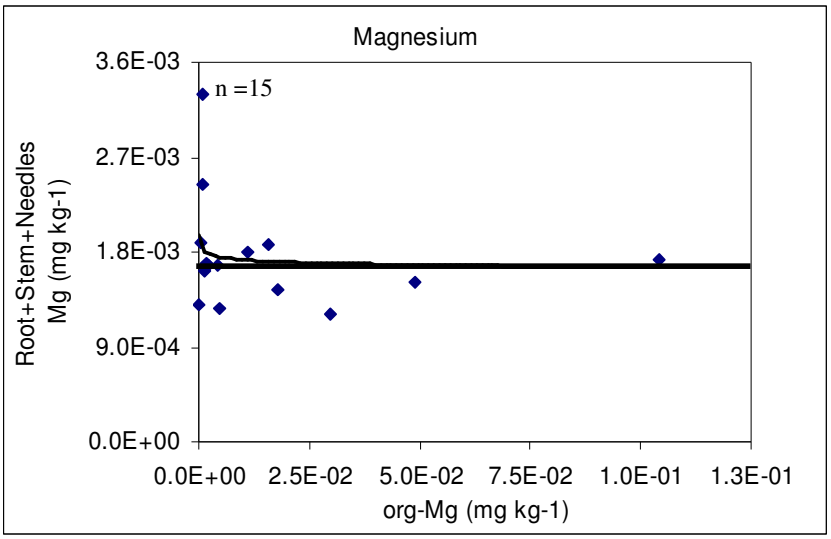


Figure 27. Magnesium concentration in whole plant (plant-Mg) to the magnesium availability as LMW carboxylate complex (org-Mg) in mycorrhizal experiment (n =15). (Mean = $1.7E^{-03}$).

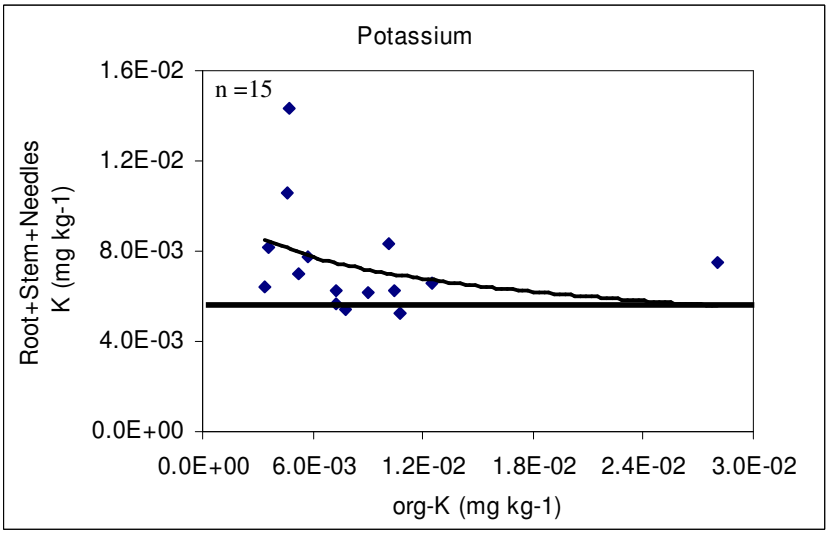


Figure 28. Potassium concentration in whole plant (plant-K) to the potassium availability as LMW carboxylate complex (org-K) in mycorrhizal experiment (N=15). (Mean = 6.3 E^{-03}).

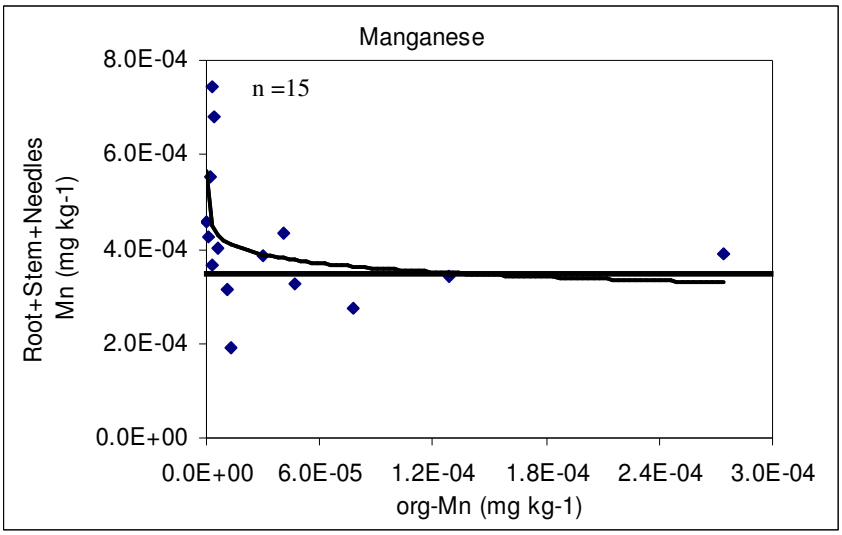


Figure 29. Manganese concentration in whole plant (plant-Mn) to the manganese availability as LMW carboxylate complex (org-Mn) in mycorrhizal experiment (n =15). (Mean = $3.4E^{-04}$).

For the non-mycorrhizal experiments the cations were calculated as LMW carboxylate-cation complexes (Appendix 6; NM1 to NM10) and were compared between the plant accumulated cation to the corresponding element in the rhizosphere soil solutions as conceptual models (Fig. 30 to 33).

The comparison of plant element concentration to the availability of the cation concentration in the substrate describes the conditions devoid of mycorrhizal fungal treatment. In the conceptual models, the highest element concentrations in the rhizosphere were compared to the mean element concentrations in plant. In the non-mycorrhized experiments the availability of Ca, Mg and K in the rhizosphere soil solutions was more than the mean element concentrations in the plant (Fig. 30; 31 and 32), suggesting high availability of these elements. With regards to Mn, the highest concentrations in the rhizosphere solutions were very low than the concentration of Mn in plant, suggesting low availability of Mn element in the rhizosphere solutions (Fig. 33).

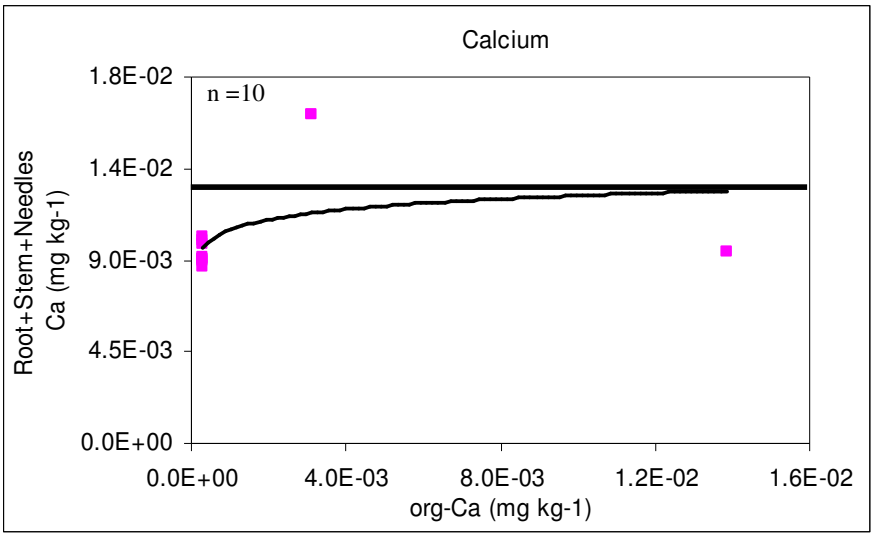


Figure 30. Calcium concentration in whole plant (plant-Ca) to the calcium availability from LMW carboxylate complex (org-Ca) in non-mycorrhizal experiment (n =10). (Mean = $1.3E^{-02}$).

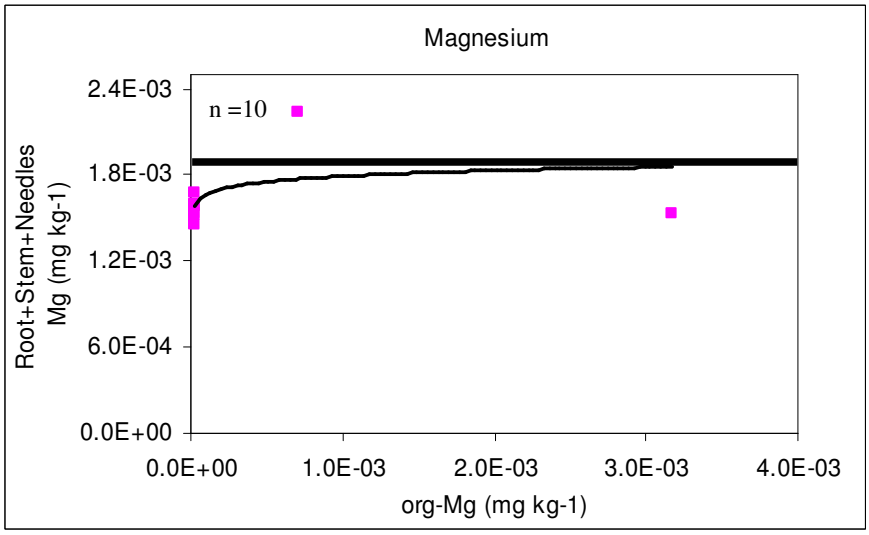


Figure 31. Magnesium concentration in whole plant (plant-Mg) to the magnesium availability from LMW carboxylate complex (org-Mg) in non-mycorrhizal experiment (n =10). (Mean = $1.9E^{-03}$).

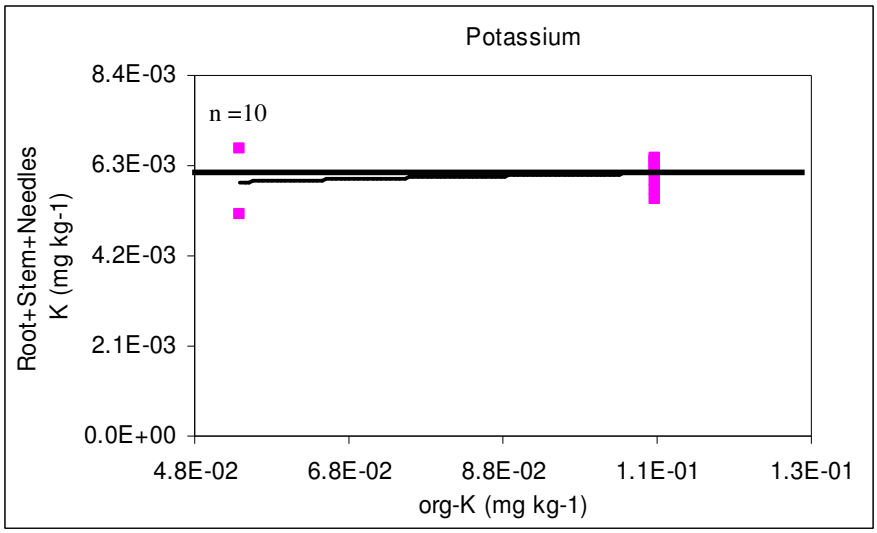


Figure 32. Potassium concentration in whole plant (plant-K) to the potassium availability from LMW carboxylate complex (org-K) in non-mycorrhizal experiment (n =10). (Mean = $6.0E^{-03}$).

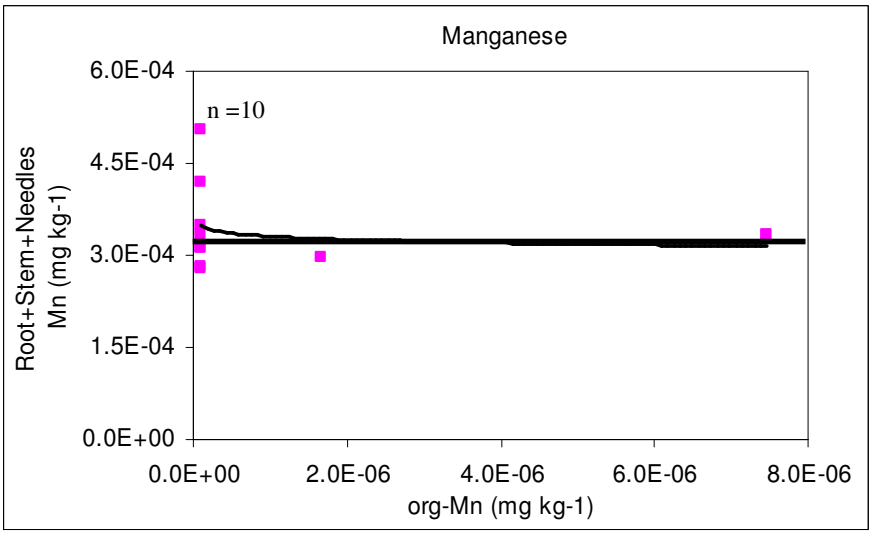


Figure 33. Manganese concentration in whole plant (plant-Mn) to the manganese availability from LMW carboxylate complex (org-Mn) in non-mycorrhizal experiment (n =10). (Mean = $3.1E^{-04}$).

4. Discussions

4.1 Experimental approach

Exudation of organic acids was reported by earlier researches (Marschner 1995, Cromack et al. 1979; Malajczuk and Cromack 1982; Lapeyrie 1988; Snetselaar and Whitney 1990; Jones et al. 1992; Rasanayagam and Jeffries 1992). Most studies on root exudation have been carried out with seedlings grown in hydroponic cultures or in quartz sand (Schönwitz and Ziegler 1982, Schwab et al. 1983); Wittenmayer and Gransee 1992). Depending on plant species, age and environmental conditions, the exudates can account for up to 40% of the dry matter produced by plants (Lynch and Whipps 1990). For a better understanding of exudates and their reaction kinetics under semi sterile conditions, miniaturization of the experiments was necessary. The growth of seedlings in petridishes (Fig. 2) was to increase quality and quantity of the analyte for analysis. At the time of germination of Scots pine seedlings; the source of phosphorus nutrient component in agar medium was modified by replacing KH_2PO_4 with $KHSO_4$. The purpose of this modification was to grow the seedlings under phosphate stress. Only those seedlings that were able to survive the P stress were used for the experiments. For subsequent growth of seedlings in quartz sand P was applied to the Hoagland nutrient solution. Quartz sand was chosen as growth substrate for our final experiments as studies done by other authors with similar microcosm investigations have identified a rise in pH of the soil solution percolate in comparison to the nutrient solution supplied during their analysis, because of use of glass beads as growth substrate (Sandnes and Elhduset 2003). To achieve a minimum destruction, only low volumes of solutes were extracted from the rhizosphere and the time span from sampling to analysis was kept as short as possible. The monitoring and handling of the experimental petridishes was easier by growing seedlings in light-temperature controlled incubator (Fig. 3). Hence, the experimental conditions were same for both mycorrhizal and non-mycorrhizal experiments.

For analysis of LMW carboxylates two techniques were used, i.e. HPLC (section 2.8.5.2) and CE (section 2.8.5.1). In studies done by other researchers, HPLC techniques have been commonly used in the analysis of LMW carboxylic acids most extensively (Tani et al. 1993; Ström et al. 1994; Krzyszowska et al. 1996; van Hees et al. 1999). CE techniques have been applied previously by other researchers to analyze LMW carboxylates (Göttlein and Blasek 1996; Bjarne et al. 1999; van Schöll et al. 2005). In the studies, it was observed that, the larger sample injection volume needed for HPLC helps in better separation of exudates when concentrations of exudates vary highly. On the contrary, it is a drawback of CE that low sample injection volume reduces the possibility to analyze complete spectra of exudates in the rhizosphere solutions. It turn out that high concentrations of specific

analyte displaces ions that occur at low concentrations (Krauskopf and Bird 1995). Hence, the detection of ions at low concentrations is strongly limited.

4.2 Ectomycorrhiza

Mycorrhiza is a structure formed by the root-fungus partnership (Trappe 1987; Read 1991). Smith and Read (1997) defined mycorrhiza as "a symbiosis in which an external mycelium of a fungus supplies soil derived nutrients to a plant root". Mycorrhizae are distinguished based on structural entities such as arbuscules or hyphal coils in arbuscular (AM) mycorrhiza, Hartig net in Ectomycorrhiza (ECM) and pelotons in orchids. The ECMs occur often at sites poor in soluble nutrients and where accumulated litter occur on soil surface. They are common symbiotic associations in trees and shrubs. Ectomycorrhizal fungi differ in their capacity to exude LMW carboxylates (Wallander and Wickman 1999; Ahonen et al. 2000; Arvieu et al. 2003; Casarin et al. 2003). It is also reported that upto 60% of the total ECM roots tips were found in the mineral soil layer, and that there was a distinct distribution pattern of ECM species within the soil profile, with some species occurring in either the mineral or the organic layer (Landwweert et al. 2003; Rosling et al. 2003). In boreal forest, trees obtain nutrients through the sheath (mantle) of ectomycorrhiza fungal tissue that envelope the fine root-tips (Fig. 34a) of the host plant members such as Pinaceae, Fagaceae, Betulaceae, Myrtaceae, Leguminosae, and also include some monocotyledons and ferns (Wilcox 1996). The ectomycorrhizal roots mostly have characteristic appearance with mantle hyphae which penetrates between the epidermal and cortical cells of the root-tip to form a ramifying structure (Hartig net) (Fig. 34b). This structure is considered as a site of fungal nutrient transfer in exchange for plant photosynthates. The mantle is connected to the environment through a hyphal network that penetrates the soil. This morphological feature greatly increases the absorption surface of the roots (Smith and Read 1997). The ECM roots provide in this way, sites for 1) nutrient and carbon transfer between the symbionts and 2) host-protection against pathogens, environmental pollution and heavy metals. However, numerous laboratory experiments have pointed out the significant interspecific variation between ECM fungi with regard to nutrient uptake and transfer, carbon demand, and their protective role (Smith & Read 1997). Mycorrhiza fungi are found to be involved in the uptake of nutrients in exchange for carbon from host plants, and transfer carbon and nutrients between plants.

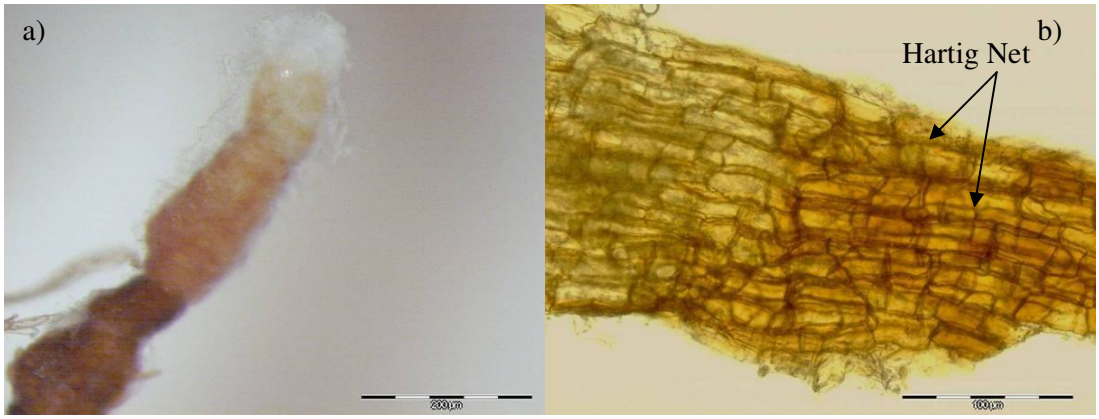


Figure 34. a) Mycorrhizal root tip surrounded by hyphal mycelia (magnification: x250; Scale: 200µm).
b) Longitudinal cross-section of mycorrhizal root tip showing Hartig Net (arrows) formed between the root cells (magnification: x500; Scale: 100µm).

Correlations between organic acids (LMW carboxylates) and uptake of the dissolved elements from the rhizosphere solutions by Ectomycorrhizal colonization are not clear (Leyval and Berthelin 1991). Ectomycorrhizal fungi have a kind of mechanisms to access nutrients altering the soil physically (Simard et al. 1997e) and causing changes in the substrate having mycorrhiza infested roots or 'mycorrhizosphere' (Robinson et al. 2003). ECMs are particularly important for tree growth in regions with low nutrient or low water status. ECM usually forms between fine roots and dikaryotic mycelia of different Basidiomycetes genera, but some may also belong to Ascomycetes genera (Smith et al. 2003). Both the ECM plants and fungi are generally able to form symbiosis with different species (Molina et al. 1992). In contrast to other mycorrhizal types, the ectomycorrhizal hyphae do not form intracellular structures (Peterson and Farquhar 1994). Ectomycorrhizal fungi are largely associated with the forest tree species e.g., *Pinus* or *Fagus*, and are especially associated with the acidic soils (Lapeyrie et al. 1990).

The important factor for the stability of ectomycorrhizal associations is the putative dependence of the fungus on the host for photosynthate (Hibett et al. 2000). The view that ectomycorrhizal fungi are obligate symbionts is based on the slow growth of fungi in pure culture and their inability to derive adequate nutrition for the growth from complex organic compounds (e.g., cellulose). The net process of ability of mycorrhizal symbiosis to utilize proteins by obtaining N from the polymers and by assimilating carbon skeletons reduces the drain upon host photosynthate (Abuzinadah et al. 1986). The proteolytic capability of mycorrhiza to decompose lignin holocellulose and lignocellulose (Trojanowski et al. 1984) implies that mycorrhizal fungi are effective competitors compared to decomposers.

Similar observations were reported earlier, that the activity of decomposer organisms is inhibited in the presence of mycorrhizal fungi (Gadgil and Gadgil 1975). Fungal hyphae can directly protect the plant roots from Al toxicity by accumulating it in its hyphae (Wilkins 1991).

Duponois (1992) hypothesized that mycorrhization helper bacteria (MHB) could soften both cells walls and middle lamella between the cells of the root cortex by means of enzymes causing the penetration easier. These specific MHB may stimulate ectomycorrhizal formation in conifer nurseries and in certain cases these bacteria eliminate the need for soil fumigation (Garbaye 1994). Infection of plants by symbiotic micro organisms may occur either by their own enzymes or by those of associated micro organisms. The significance of these enzymes for mycorrhization and the growth of the mycelia was stressed by Leake and Read (1989). It is reported in the work by Dahm and Strzelczyk (1995) that all ectomycorrhizal fungi, such as *Laccaria laccata* and *Pisolithus tinctorius* studied, produced exoglucanases. However, *Pisolithus tinctorius* was also found to acidify the mycelium during growth on modified Melin-Norkans (MMN) agar medium (Cairney and Ashford 1991), and it is possible that a similar lowering of pH in the vicinity of mycorrhizal roots may play a role indirectly by increasing the effectiveness of reducing agents or otherwise changing the properties of the rhizosphere to favour reducing activity (Bienfait 1988). Thus, experiments in semi-sterile conditions may contain such MHB and may be present in our experiments, however, have not been investigated.

4.3 Growth dynamics of *Pinus sylvestris* L. seedlings

To study the effect of treatments, investigations were started with higher number mycorrhizal seedlings (n = 75) than non-mycorrhizal seedlings (n = 50) to avoid reductions in relative growth rate due to mycorrhizal infections (Ingestad et al. 1986). At the end of the experiment duration, the survival percentage calculated was 82% for non-mycorrhized variant and 75% for mycorrhized variant. The objective of more individuals per petridish in both variants was to increase the rooting density, such that, a high concentration of the exudate is achieved for a small amount of extracted sample. The distribution of *Pinus sylvestris* species, in greenhouse experiments shows inequal growth of individuals depending on the spatial factor and robustness of the seedlings in individual petridish of mycorrhizal (Fig. 7a) and the non-mycorrhizal experiments (Fig. 7b). The above and below ground distribution also varied per seedling (Fig. 8a and Fig. 8b). To describe the inequal distribution of growth, root weight of seedlings were plotted as a histogram and compared, indicating a normal distribution for non-mycorrhizal experiment (Fig. 9a) and a skewed distribution for mycorrhizal experiment (Fig. 9b).

The non-uniform distribution can be compared on the basis of Lorenz curves by taking into account the cumulative root weight proportion of seedlings (Fig. 10). As the Gini coefficient ($\overline{G}$) is a measure of inequality among individuals of a population, it reflects dominance/suppression relationships. The degree of the dissimilarity between the non-mycorrhizal and mycorrhizal treatments can be expressed by the Gini coefficient. The Gini coefficient for non-mycorrhizal seedlings was lower ($\overline{G}$ =0.32) than mycorrhizal seedlings ($\overline{G}$ =0.41), indicating an inequal distribution of individuals in the two variants. The above findings reflect high nutrient foraging activity by dominant individual plant root system, causing the dependence of other less competitive plants to be less active in a petridish. Hence, seedlings belonging to a petridish were compared rather than a single individual seedling for the treatments.

The root tissue density (RTD) and specific root length (SRL) (section 3.3) contributed by the non-mycorrhizal and mycorrhizal seedlings was not significantly different. Likewise, SRL is an indication of relationship of root length in proportion to the given mass of roots (Robinson et al. 1999). Correlationship between the increases in SRL to decrease in inorganic Phosphate supply was shown in other plant studies (Schroeder and Janos 2005). Root traits such as high root tissue density (RTD) are considered to show functional significance to the root longevity, resistance to droughts etc., (Roumet et al. 2005); specific root length (SRL) is generally associated with high respiration rate, reflecting metabolic activity and is related to uptake of nutrients and assimilation (Tjoelker et al. 2005). Other studies on woody species, reported that the patterns in morphological traits (SRL and root diameter) between fast and slow growing individuals were similar for seedlings of same species, grown either in laboratory or field, while this was not the case for root physiological traits (phosphorus uptake and root respiration) (Comas et al. 2002; Comas & Eissenstat 2004). A number of studies have related leaf traits between annual and perennial species and found differences (Garnier 1992; Gross et al. 1992; Garnier and Laurent 1994; Garnier and Vancaeyzeele 1994). Roumet et al. (2005) have found in their studies that root traits follow similar patterns to those observed for leaf traits. Also, both the leaves and roots of annuals had higher N concentration and specific root area or length (SRL) and lower root tissue density (RTD) and thickness/diameter. It is found that, there are positive relationships between leaf traits and root parameters such as, N concentration, root tissue density (RTD)(Ryser 1996; Craine et al. 2001; Craine and Lee 2003), longevity and respiration rate (Tjoelker et al. 2005). However, contrastingly other researches reported SRL and specific leaf area (SLA) to be weakly related or not related to each other at all (Tjoelker et al. 2005). Hence, applicability of these relationships remains still uncertain. However, in our experiments, comparing the needles of Scots pine, no significant difference in leaf area ratio (LAR) was observed in non-

mycorrhizal and mycorrhizal seedlings. Therefore, the photosynthetic activity of the needles is assumed to function equally in both the treatments.

Depending on the physiological traits and the existing environmental parameters in the rhizosphere solutions, such as, pH, nutrient elements and LMW carboxylates, the variation in the treatments was determined by applying principle component analysis (PCA). Using PCA, 86.5% of the total variation in the non-mycorrhizal and mycorrhizal experiments was found. The upper two quadrants in PCA plot were occupied by petridishes belonging to the mycorrhizal seedlings and the bottom two quadrants was corresponding to the non-mycorrhizal seedlings (Fig. 22). Comparing the carbon flux with regards to C accumulation in the roots (Tables 5a&b) and leaves (needles) (Tables 6a&b) of non-mycorrhizal and mycorrhizal seedlings shows loss of photosynthates for the maintenance of symbiotic relationship with ectomycorrhizae for the mycorrhizal treatment (Snellgrove et al. 1982). However, this loss in photosynthates may be compensated for by increased photosynthesis in the mycorrhizal treatment basing on higher C: N values (Tables 6a&b) in the leaves (Gianinazzi-Pearson and Gianinazzi 1983). It is found that, by using ^{14}C labelling, 17% of radioactivity was recorded to be assigned to fungal mycelia (Wu et al. 2002). This sort of carbon flow suggests that fungal mycelium is an important sink for photosynthates as a result of C allocation in ectomycorrhizal seedlings. It is also found that roots can control the amounts of LMW carboxylates accumulation in the rhizosphere by two mechanistic approaches, such as, those regulated by C efflux and those regulated by C influx. However, the roots attempt to recapture the organic acids (C influx) will be smaller than the C efflux, as the regulation depends directly on the plasma membrane's electrochemical potential gradient, which is the driving force for such transport. Thus, the C loss can be attributed to the unidirectional efflux (Farrar and Jones 2003). The effectiveness of treatments in growth was distinctly attributed to the differences in physiological aspects of seedlings.

4.4 Biomass distribution of seedlings of *Pinus sylvestris* in mycorrhizal and non-mycorrhizal treatments

For the calculation of seedling biomass grown in sand matrix, the sand particles adherent to roots (Fig. 35) give an erroneous interpretation to the actual dry mass contributed by roots. Determination of root dry weight along with the binding of quartz sand impedes the calculation of accurate root weight (van Schöll, et al. 2006). Also, with regards to the element content in the root tissues, it is important to measure accurate root weight devoid of sand substrate. This was resolved by applying procedures to estimate the weight of excess quartz sand in the dry root tissue (section 2.8.1). Comparison of dry plant biomass of both the mycorrhizal and non-mycorrhizal seedlings was done based on cumulative weight proportion owing

to difference in the number of petridishes. Studying the biomass relationships in this form was ideal for comparison.

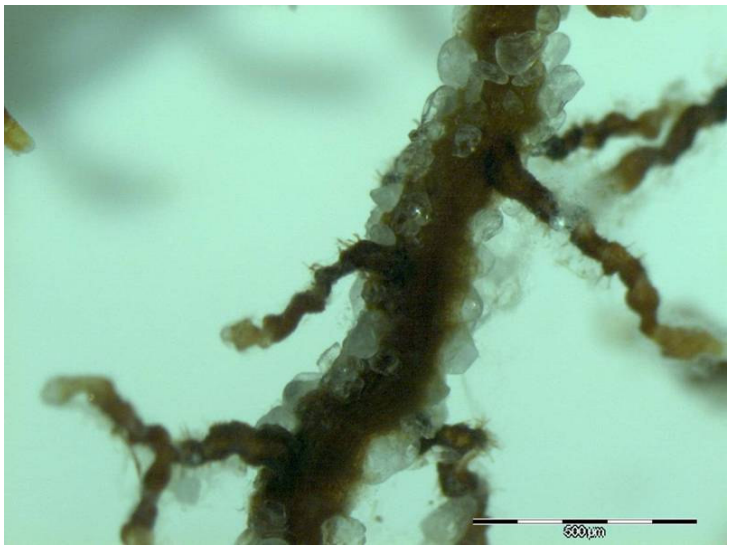


Figure 35. Mycorrhizal root tip of *Pinus sylvestris* L. adhered with quartz sand substrate (magnification: x10; Scale: 500µm).

Though there was inequal growth pattern (Fig. 6a & 6b) between the individuals of the treatment, the contribution of biomass for each experimental petridish was same depending on equal nutritional supplements and spatial criteria as a controlling factor. The reason for the inequal root growth dynamics influenced by ectomycorrhizal fungi in our experiments may be because of initial induction of lag root growth (Norby and Jackson 2000). A biomass accumulation with 10% increase in non-mycorrhizal seedlings was observed compared to biomass of non-mycorrhizal seedlings. The reductions in plant biomass of mycorrhizal seedlings can be attributed to the mycorrhizal infections (Ingestad et al. 1986; Kähr and Arveby 1986; Nylund and Wallander 1989; Dosskey et al. 1990) and would occur even at low infection percentages (Strenström and Ek 1990).

4.5 Root exudates

The types of LMW carboxylates found in the rhizosphere soil solutions as main organic substances were oxalate/oxalic acid, citrate/citric acid, malate/malic acid, succinate/succinic acid and lactic acid according to analysis by HPLC/CE. The mean concentrations analyzed for non-mycorrhizal and mycorrhizal seedlings for oxalate was upto 1965 µM (NM) and 2277 µM (M); for L-malic acid was upto 26 µM (NM) and 32 µM (M); for lactic acid was upto 121 µM (NM) and 73 µM (M); for citric acid was upto 30 µM (NM) and 18 µM (M). But compared to oxalate

other LMW carboxylate concentrations were found to be very low in both the treatments (Tab. 7). However during the experiments it was found that the concentrations of LMW carboxylates were fluctuating. Similar studies focusing on relationships between mineral effects on mycorrhizal and non-mycorrhizal seedlings of *Pinus sylvestris* have identified malic, citric, oxalic and formic acids in soil solutions at concentrations of upto 20µM with a variation between the replicate samples to be high (Wallander and Wickman 1999). In the experiments higher concentrations of lactic acid were found only in the initial sample period (month 3) with drastic reductions towards the end of the investigation. The initial high concentrations of lactic acid during the sampling period of exudate may be due to product of root debris decay (Jones 1998).

Table 7. Comparison of mean concentrations of LMW carboxylates analyzed in the non- mycorrhized (n = 10, mean ± SD) and mycorrhized variants (n = 15, mean ± SD).

	Oxalate (µmol L-1)				L-malic acid (µmol L-1)				Lactic acid (µmol L-1)				Citric acid (µmol L-1)			
	Variants				Variants				Variants				Variants			
	NM		M		NM		M		NM		M		NM		M	
Date of exudate Sampling																
Month 3	475	(±44)	1236	(±201)	5	(±1)	32	(±5)	121	(±52)	73	(±10)	30	(±23)	5	(±2)
Month 3	773	(±129)	993	(±136)	26	(±6)	17	(±5)	82	(±53)	20	(±5)	25	(±13)	18	(±7)
Month 4	1337	(±198)	1234	(±186)	4	(±2)	2	(±0)	10	(±1)	12	(±1)	3	(±1)	2	(±0)
Month 8	1965	(±108)	2277	(±150)	1	(±0)	--	--	11	(±1)	17	(±5)	2	(±1)	2	(±0)
NM=non-mycorrhized; M = mycorrhized																

Roots may influence the soil solution chemistry in the rhizosphere, by the uptake of water and ions and the exudation of organic compounds (Jungk and Claasen 1986; Marschner et al. 1986). It is reported that in majority of the seedlings, citrate and malate are the principle organic anions accumulated in the plant root, due to deprivation of phosphorus in soils. Mostly, phosphorus is one of the limiting factors for the plant growth (Neumann and Römheld 1999). It is also reported by studies done on Al stress on plants like maize and wheat, root exudations of specific carboxylates (e.g., oxalate, citrate, malate and succinate) are released for the uptake of nutrition by way of mobilization and immobilization of the constituent complexes in the soils (Pellet et al. 1995; Ryan et al. 1995; Zheng et al. 1998). Sometimes these exudations are released in high amounts because of deficiency of nutrients or Al toxicity or as a natural process of diffusion.

4.6 Release of exudates (Low Molecular Weight carboxylates)

In the experiments, the release of exudates in non-mycorrhizal seedlings can be attributed solely to the roots of the *Pinus sylvestris* L., whereas, in mycorrhizal seedlings the increase in the concentrations of LMW carboxylates (Fig. 18) may be derived from both the root and the ectomycorrhizal partner. Other researches also reported that tree species colonized by ectomycorrhizal fungal symbionts exuded more exudate (oxalate) than non-mycorrhizal tree species (Ahonen et al. 2000). Higher concentrations of organic acids or more complex suite of acids have been found in rhizospheric soil than in the bulk soil (Grierson 1992; Griffiths et al. 1994). Root exudates also contain chemical molecules governing the development of plant-microbe symbiosis by providing powerful signals, which in turn alert the mycorrhizal fungi to the presence of host plant (Felix and Phillips 2000). Root exudation and chelating LMW carboxylates increases the availability of nutrient cations in the rhizosphere of plants and fungi (Marschner and Römheld 1996). As the chemical conditions found in the rhizosphere are different, it has been postulated that rhizosphere, rather than bulk soil solution needs to be considered in order to evaluate nutrient availability and soil acidity effects on tree roots (Løkke et al. 1996). The production of LMW carboxylates occurs in the mitochondria of the cells and is the by-product released during the metabolic cyclic pathway, also known a Tri-Carboxylic Acid (TCA) cycle into cytoplasm (Gadd 1999; Neumann and Römheld 1999). Based upon the fact that the concentration of organic acids in the cytosol (ca. 0.5-10 mM) is about 1000 fold higher than that present in the soil solution (0.5-10 µM); theoretically, the release of LMW carboxylates must be occurring always (Jones 1998). Due to operation of ATP-driven pumps (H^+ - ATPases) and the large cystolic K^+ diffusion potential, a substantial electric charge gradient exists across the plasma membrane (Samuels et al. 1992). As the H^+ is expelled into the apoplast by these H^+ - ATPases creating a charge gradient to facilitate the uptake of cations from the rhizosphere soil solution, it also tends to draw anions (e.g., $citrate^{3-}$, $malate^{2-}$) out of the cells and into the external soil solution (Jones 1998).

In this study oxalate was found to be in higher concentrations with respect to other LMW carboxylates in the rhizosphere soil solutions (Fig. 20). In comparison to LMW carboxylates analyzed in the rhizosphere soil solutions, oxalate ($p = 0.01$) and L-malic acid ($p = 0.05$) were significantly higher in the mycorrhized than the non-mycorrhized variants (Fig. 21) and lactic and citric acids, however, were in a much lower concentrations for both the treatments. Succinate was not found at all.

4.7 Effect of exudates (Low Molecular Weight carboxylates)

In this study, pH values in the rhizosphere substrate solutions of mycorrhizal experiments were lower than the non-mycorrhizal experiments. However,

differences were not found to be statistically different. Lower pH values in the mycorrhizal treatments may be due to influence of LMW carboxylic acids (Haynes 1990; Jones and Darrah 1994). Similar lowering of pH in soils was reported when *Pinus sylvestris* species were inoculated with 'Suillus variegates' ectomycorrhizas, in comparison to non-mycorrhizal Pinus species (Wallander et al. 1997). Another possible explanation refers to an enhanced cation uptake rather than anions, thus, causing an efflux of H^+ from roots (Haynes 1990; Marschner 1995). Thus, it is assumed that pH decrease was caused by exudation of organic acids. In mycorrhizal experiments due to increased exudation of the oxalic acid may lead to low pH measurements in the rhizosphere soil solutions with the pH in the range of 4.8 to 5.5. Conversely, the pH values in non-mycorrhizal experiments were in the range of 5.5 to 6.3 probably due to nitrate present in the rhizosphere soil solutions. The influence of nitrate as illustrated by PCA plot (Fig. 24) shows good correlation with non-mycorrhizal petridishes and indirectly, also influencing the substrate pH to be higher (Marth 1995). Nitrate was seldom found in the petridishes of mycorrhizal experiments in comparison to non-mycorrhizal experiments. In studies with Norway spruce (Schneider et al. 1989), show that acidity at soil-root interface was increased due to enhanced cation uptake, causing nearly equivalent exchange of protons from the roots. The greenhouse investigation of exudates of LMW carboxylates should be interpreted cautiously in comparison to field situations. As the cations, especially protons (H^+), excreted along with LMW carboxylates into the rhizosphere may lead to maintain charge balance, causing acidification (Hinsinger 2001a and Hinsinger et al. 2003). However, carboxylate exudation may not be invariably associated with acidification in field studies (Roelofs et al. 2001). As under field conditions biological and physical factors play an important role in the formation of inactive organo-metallic complexes of toxic metals, such as, Fe and Al (Lambers et al. 1998). The formation of such inactive forms is due to decomposition of the organic substance acting as a cementing component between the metals, the process termed as mineralization (Merckx et al. 1987). LMW exudates are also supposed to enhance mineral weathering (Drever and Stillings 1997). If acidified soil conditions prevail it may lead to immobilization of P and may correspondingly, influence the extent of ionization of LMW carboxylates (Jones 1998; Hinsinger et al. 2003). Hence, reducing the chelating ability of LMW carboxylates and potentially rendering them ineffective (Pearse et al. 2006).

Generally, in mycorrhizal seedlings, the symbiotic association enhances the rate of nutrient uptake (Barber and Martin 1976). In contrast to this notion, no significance differences were found in the accumulation of major cations between the treatments, other than Mn^{2+} ($p = 0.063$) and Fe ($p < 0.05$) in the dry weight of the seedlings (Fig. 15). Several plants are known to respond to increase exudation of LMW carboxylates when grown under P deficiency (Ryan et al. 2001). Wouterlood et al. (2005) suggested that LMW carboxylate exudation has become

an inherent trait of plants that are adapted to low-P environments. Also, numerous studies of rhizosphere communities have shown that the micro organisms in the substrate play key roles in assimilation of plant nutrition (Lynch 1990; Paris et al. 1995 1996). Hence, the nutrient status of needles of seedlings (Tab. 8a) with the standard element status in the chart (Table 8b) shows P deficiency in both the mycorrhizal and non-mycorrhizal seedlings.

Table 8a. Nutrient status of seedlings needles at the end of experiments.

Element	Variant	
	mycorrhized	non-mycorrhized
P (mg/g DW)	Deficient	Deficient
K (mg/g DW)	Excess	High
Ca (mg/g DW)	Excess	Excess
Mg (mg/g DW)	Excess	Excess
Mn (mg/g DW)	Adequate	Adequate
Fe (mg/g DW)	Adequate	Adequate

Table 8b. Standard nutrient status in needles of pine tree seedling at 1st Needle Year (modified after AID 1991; Block et al. 1991 and Hüttl 1992). Source: Forstliche Standortsaufnahme 2003.

Element	mg/g dry matter				
	Deficient	Low	Adequate	High	Excess
P	<1.2	1.2 – 1.4	1.4 – 1.8	1.8 – 2.0	>2.0
K	<3.5	3.5 – 4.0	4.0 – 5.0	5.0 – 7.0	>7.0
Ca	<2.0	2.0 – 2.6	2.6 – 3.3	3.3 – 4.0	>4.0
Mg	<0.75	0.75 – 1.0	1.0 – 1.25	1.25 – 1.5	>1.5
Mn	<0.05	0.05 – 1.0	1.0 – 2.0	2.0 – 4.0	>4.0
Fe	<0.02	0.02 – 0.05	0.05 – 0.1	0.1 – 0.5	>0.5

Nevertheless, phosphorus ($p < 0.05$) uptake in mycorrhizal treatments was found to be significantly higher (Fig. 15). Nutrient mobilization is partly affiliated to exudation for the availability of the nutrient uptake by seedlings for its metabolic purposes. Previous studies on dynamics of transport and nutrient uptake models for growing plants is found in the literature (Hoffland et al. 1992), but has limitations of over estimation of nutrient uptake. Plants possess highly specialized mechanisms to stimulate metal bioavailability in the rhizosphere, and to enhance uptake into roots (Römheld and Marschner 1986). Along with the solute effect on root water uptake (considered to be a function of the total solute concentration or osmotic potential of the soil solution), the uptake of specific nutrients will depend on the specific ion concentration in rhizosphere solutions (Bouldin 1988).

4.8 Conceptual model for comparison of plant cation to LMW carboxylate complexed cation in the rhizosphere soil solutions of both the experimental variants

Though the plant available free cations analyzed by CE instrument using Metol complexing buffer (Tab. 2) may be estimated in the rhizosphere soil solutions (Baumann 2004), other forms of plant available cations mobilized as organo-metallic compounds (LMW carboxylate-cation complexes) would be another possibility of nutrient availability for the seedlings.

Although the basic equations governing nutrient uptake in hydroponics have been in use for several decades at the level of roots and root systems (Nye and Tinker 1977; De Willigen et al. 2000), the specific effects of plant rhizosphere modification are still under research (Kirk 2001; Claassen 2001). Applications to intraspecific competition or long term effects of such modification are still relatively scarce, depending on the binding to soil particles and the biological availability of the active compound, such as, effects on P may carry over to a subsequent crop. (Hocking and Randall 2001; Gransee 2001; Lu et al. 2001). Previous studies show comparison of organically bound cation, for e.g., Al, done by using MINEQL+ chemical equilibrium modelling tool to the inorganic form of cation present in the soil solutions (van Hees and Lundström 2000). The objective of the above studies seems to emphasize the availability of the LMW carboxylate complexed cation to the total cation concentrations in the soil solutions, as calculated by the model.

In this study, the relationship between the possible cations formed as organo-metallic complexes in the rhizosphere soil solutions to the plant nutrient concentrations, are demonstrated with a conceptual model. According to Kirk (2002), “it is necessary to incorporate empirical inputs into model for it is not possible to describe biological systems in purely mechanistic terms”. Hence, in the conceptual model, the analyzed plant nutrient element content is transformed into concentration, and the corresponding cation in the rhizosphere soil solution is estimated by calculating the organometallic complexes (details in section 3.10), depending on the analyzed LMW carboxylates for both the treatments (as shown in the Fig. 26 - 29 for mycorrhizal and Fig. 30 - 33 for non-mycorrhizal treatments). For the calculation of the organometallic complexes - PHREEQC, a geochemical modelling software tool was used. Most of the nutrients (Ca, Mg and K) analyzed in the seedlings were not significantly different. As the elements, such as, Mn ($p = 0.063$) and Fe ($p<0.05$) were found to be higher in mycorrhizal seedlings, the status of cation availability in rhizosphere was checked using the conceptual model. Comparing the conceptual model especially for Mn, the maximum nutrient (Mn) availability in mycorrhizal treatments was found to be higher than in non-mycorrhizal treatments. From the conceptual model, crucial information of

probable limiting or non-limiting conditions in the rhizosphere soil substrate can be interpreted. For example, by comparing the total plant nutrient (Mn) concentration, i.e., Mean = $3.4E^{-04}$ mg kg^{-1} is larger than maximum substrate concentration of $2.7E^{-04}$ mg kg^{-1} for mycorrhizal treatment (Fig. 29), and the Mean = $3.1E^{-04}$ mg kg^{-1} is larger than maximum substrate concentration of $7.5E^{-06}$ mg kg^{-1} for non-mycorrhizal treatment (Fig. 33), inferring limiting conditions. For cations, such as, calcium (Fig. 26), magnesium (Fig. 27) and potassium (Fig. 28) of mycorrhizal treatment, and calcium (Fig. 30), magnesium (Fig. 31) and potassium (Fig. 32) of non-mycorrhizal treatment, the mean total plant nutrient concentrations are lower than the maximum substrate concentrations, inferring non-limiting conditions.

Thus, from the conceptual models it was useful to identify the low availability of Mn nutrient in the substrate, though the cation nutrient status in the seedlings showed no deficiencies.

Though, deficiency of phosphorus in the pine needles (comparing Table 8a and Table 8b) can be attributed to low availability in substrate, it is a disadvantage of conceptual model in evaluating phosphate (anion) availability in the rhizosphere.

As the conceptual model is based on studies in artificially supplemented nutrient system, applicability of it should be tested under field conditions.

5. Conclusions

As ectomycorrhizal fungi are more commonly found in podzolic soil than non-podozols, the influence of ectomycorrhiza on the release of exudates in *Pinus sylvestris* (Scots pine) are investigated in this thesis work. The complete volume of the petridishes with the substrates is termed as the rhizosphere/mycorrhizosphere depending on the non-mycorrhized or mycorrhized variant. As the conditions in the petridishes are maintained homogenously, influence of external factors is considered very negligible depending on the results obtained. The mycorrhization of *Pinus sylvestris* was achieved in the range of 90% to complete, for green house experiments in semi sterile conditions. For the purpose of mycorrhizal infection of the plants and to reduce the influence of allelopathic interactions between the fungal species, only one type of ectomycorrhizal fungi was used. The significance of influence of mycorrhization on production of exudates of low molecular weight carboxylates was found be higher in mycorrhized variants than in the non-mycorrhized variants. The inequal root growth dynamics influenced by ectomycorrhizal fungi in our experiments may be because of induction of lag root growth initially. This may be a crucial factor for consideration during root growth studies. Hence, this information is useful for consideration in the studies of growth dynamics involving mycorrhizal species.

The method development, analysis of LMW carboxylates on capillary electrophoresis and high performance liquid chromatography was successfully accomplished. Given the advantages of the usage of the capillary electrophoresis for low sample volumes; it was a drawback when concentration of any single carboxylate substance in the analyte is too high in comparison to the other substances. Hence, analysis was done on both the instruments to compensate for drawbacks in either of them.

The contribution of excess weight by the quartz sand adherence to roots was resolved because of necessary modification in the procedure for digestion techniques normally followed for total cation analysis. This modification was also important for other analysis depending on weight of the plant material, such as, biomass and for recalculation of C, N and S measurements.

The elemental analysis of quartz sand free dry weight of plants shows no significant differences between the accumulation of nutrients (Ca, Mg and K) between mycorrhized plants and non-mycorrhized plants. In mycorrhizal seedlings higher uptake of iron, phosphorus and manganese are found due to symbiotic association with ectomycorrhizal fungi.

The conceptual model provides additional information about the availability of cations (which are supplied as nutrients) to the accumulated cations by seedlings.

Though the pine needle concentrations of cations (Ca, Mg, K and Mn) were not deficient, low availability of manganese was found from the conceptual model. Similarly, other cations such as calcium, magnesium and potassium were available in large concentrations in soil solutions

But estimation of phosphate availability in rhizosphere soil solution is a drawback of conceptual model.

All the experiments were done in a controlled environment to provide us with the information that can be applied to the studies in nature without natural soil conditions.

As only, the exudation of LMW carboxylates is focused on here, future studies with regard to the phytosiderophores dependent species in restoration of disturbed landscapes would be an added advantage.

6. Summary

The objective of this research emphasizes on characterization of the possible the Low Molecular Weight (LMW) carboxylates in the rhizosphere soil solution due to influence of ectomycorrhiza on root exudates. LMW carboxylates form organometallic complexes and are in a form suitable for uptake by plants for its metabolic purposes. As the nutrient sorption by the plants is by release of root exudates into the rhizosphere soil, rhizosphere soil solutions are conducive for investigation of growth of plants. For a better understanding of the characteristics of the root exudates, non-mycorrhized as well as mycorrhized plants are investigated in this study. The mycorrhization of *Pinus sylvestris* seedlings was achieved in the range of 90% to complete in our green house experiments in semi sterile conditions. To maximize the concentration of the LMW carboxylates it was useful to increase the density of the roots per volume of the space in our petridish experiments. For the purpose of mycorrhizal propagation only single ectomycorrhiza fungal strain was used in our experiments to reduce the influence of allelopathic interactions between the fungal species. For the collection of rhizosphere soil solutions sterile plastic mini suction tubes (Rhizon) were used. These plastic tubes are an advantage over using ceramic tubes as indicated by studies done earlier. The types of exudates analyzed in our samples were oxalate/oxalic acid, L-malate/L-malic acid, citrate/citric acid succinate/succinic acid and lactic acid. To identify possible organometallic complexes, labile anion concentrations of the LMW carboxylates were desired. To differentiate between respective low molecular organic acids it was necessary to develop a workable method to analyze the exudates on High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis instruments.

The adherence of quartz sand to roots is an obstacle for accurate measurements. Hence, applying the techniques followed in this work was helpful in increasing the accuracy of weight based analytical measurements. The elemental analysis of quartz sand free dry weight of plants shows no significant differences between the uptake of nutrients between mycorrhized plants and non-mycorrhized plants, except higher uptake of iron, phosphorus and manganese in mycorrhizal seedlings.

The significance of influence of mycorrhization on production of exudates of low molecular weight carboxylates was found be higher in mycorrhized variants than in the non-mycorrhized variants. With this type of experiments, it was easier to compare between the variants of treatment, especially considering the inequal growth patterns in these experiments. The results obtained from these experiments are useful to understand the growth dynamics of the plant systems and to study the influence of ectomycorrhizal fungi causing lag root growth. As the conditions in

the petridish rhizotrons were maintained homogenously, influence of external factors was considered very negligible depending on the results obtained.

The next task was to study the speciation of organometallic complexes formed between calcium, magnesium, potassium and manganese, as the nutrients supplied to the variants, and the LMW carboxylates. The major cations bound as reduced organometallic complexes (formed with LMW carboxylates) were calculated using a geochemical modelling software tool- PHREEQC. These calculations were used in developing a conceptual model. The conceptual model is an easy tool for comparison of plant-accumulated cations and corresponding cations existing in the rhizosphere soil solution. It also provides additional information about availability of the nutrients in the rhizosphere soil solution.

7. Literature

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8. Appendix

Tab. A1: Summary of Benecke-Agar for germination of seedlings (Baumann, 2004)

Chemicals	in 1 L distilled Water
$C_8H_{18}N_2O_6S_2$ (Pipes)	2,000 g
$KHSO_4$	100 mg
NH_4NO_3	200 mg
$MgSO_4 * 7\ H_2O$	100 mg
$CaCl_2 * H_2O$	100 mg
$FeCl_3$	5 mg
Micro elements after FORTIN (summarised in Tab. A2)	10 ml
0,1 N H_2SO_4 for adjusting to pH 5	
Agar	10,000 g

Tab. A2: Summary of the micro elements by FORTIN (Baumann, 2004)

Chemicals	in 1 L distilled Water
KCl	3,728 g
H_3BO_3	1,546 g
$MnSO_4 * H_2O$	845 mg
$ZnSO_4 * 7\ H_2O$	575 mg
$CuSO_4 * 5\ H_2O$	125 mg
$(NH_4)_6Mo_7O_{24} * 4\ H_2O$	18 mg

Tab A3: Hoagland-nutrient solution supplied to the plants (Schopfer, 1989; Baumann, 2004)

Chemicals	in 1 L distilled Water
KNO_3	506,00 mg
$Ca(NO_3)_2 * 4\ H_2O$	1,18000 g
KH_2PO_4	136,00 mg
$MgSO_4 * 7\ H_2O$	439,00 mg
FeNa-EDTA*	66,10 mg
H_3BO_3	2,86 mg
$MnCl_2 * 4\ H_2O$	1,81 mg
$ZnCl_2$	0,11 mg
$CuCl_2 * 2\ H_2O$	0,05 mg
$Na_2MoO_4 * 2\ H_2O$	0,03 mg

* Prior to nutrient supply process Fe-EDTA component was added.

Tab. A4a. HPLC standard curve slopes used for calculating the concentrations and correlation (R^2).

LMW carboxylates	Slope of curve	R^2
Oxalic acid	0.1883	0.9964
L-malic acid	0.0117	0.9938
Citric acid	0.0137	0.9936
Lactic acid	0.0049	0.9953
Succinic acid	0.0137	0.9998

Tab. A4b. CE standard curve slope values used for calculating the concentrations and correlation (R^2).

LMW carboxylates	R^2
Oxalate	0.90
L-malate	0.91
Citrate	0.93
Succinate	0.92

Tab. A5a. C-, N- and S- concentrations (%) of non-mycorrhizal seedlings showing before and after applying root weight corrections with correction factor in dry root mass per petridish (n =10).

Serial Number of petridish	Before root weight corrections			Correction factor	After root weight corrections			C : N
	C %	N %	S %		C %	N %	S %	
N1	23.51	0.43	0.10	3.15	35.19	0.65	0.15	56.8
N2	28.56	0.55	0.27	2.86	31.05	0.60	0.30	54.5
N3	28.30	0.49	0.17	1.50	41.97	0.73	0.25	51.8
N4	34.42	0.56	0.15	1.09	44.27	0.72	0.19	57.8
N5	30.62	0.42	<0.1	1.48	43.31	0.59	---a)	61.5
N6	33.27	0.47	<0.1	1.29	41.19	0.58	---a)	72.9
N7	23.63	0.34	<0.1	1.41	36.21	0.52	---a)	70.8
N8	30.49	0.44	<0.1	1.24	40.26	0.58	---a)	69.5
N9	25.45	0.43	<0.1	1.53	39.64	0.67	---a)	69.3
N10	33.40	0.48	<0.1	1.32	42.75	0.61	---a)	59.2
	$\overline{X} = 29.17$	$\overline{X} = 0.46$			$\overline{X} = 39.59$	$\overline{X} = 0.63$		$\overline{X} = 63.7$

Tab. A5b. C-, N- and S- concentrations (%) of mycorrhizal seedlings showing before and after applying root weight corrections with correction factor in dry root mass per petridish (n =15).

Serial Number of petridish	Before root weight corrections			Correction factor	After root weight corrections			C : N
	C %	N %	S %		C %	N %	S %	
M1	18.61	0.35	0.13	2.24	41.71	0.78	0.30	53.2
M2	16.09	0.28	0.11	2.37	38.08	0.66	0.26	57.9
M3	22.94	0.34	0.15	1.66	38.14	0.57	0.24	66.9
M4	18.90	0.32	0.14	1.88	35.49	0.59	0.26	59.9
M5	26.16	0.43	0.17	1.51	39.53	0.65	0.25	61.0
M6	9.99	0.17	<0.1	3.96	39.53	0.69	---a)	57.6
M7	25.31	0.43	<0.1	1.59	40.22	0.68	---a)	59.3
M8	10.88	0.20	<0.1	3.26	35.48	0.66	---a)	54.0
M9	15.99	0.27	0.11	2.21	35.26	0.60	0.23	58.5
M10	21.16	0.38	0.12	2.09	44.26	0.80	0.25	55.2
M11	23.22	0.48	0.17	1.79	41.53	0.86	0.30	48.4
M12	na	na	na		na	na	na	na
M13	15.02	0.22	<0.1	1.82	27.41	0.40	---a)	69.4
M14	10.19	0.19	<0.1	2.24	32.13	0.61	---a)	52.4
M15	13.14	0.23	<0.1	2.37	37.56	0.66	---a)	53.2
	$\overline{X} = 17.03$	$\overline{X} = 0.30$			$\overline{X} = 37.6$	$\overline{X} = 0.66$		$\overline{X} = 57.1$

Appendix 6 (M1) Input data for PHREEQC simulation for mycorrhized petridish

```
              M1.
             SOLUTION 1
                     temp      23
                     pH        4.94
                     pe        4
                     redox     pe
                     units    mg/l
                  K     234.77
                  Ca    189.05
                  Mg     43.29
                  Na      4.15
                  Mn     0.59
                  Fe    10.06
                  Al     0.009
                  N     136.17 as NO3
                  Cl      0.96
                  S      57.15 as SO4
                  EDTA               51.90

                  Oxalate            143.0 charge
                  Citrate              0.38
             END
-----------------------------Distribution of species-------------------------
                                                      Log       Log         Log
               Species            Molality    Activity  Molality  Activity     Gamma

Citrate                       2.011e-006
             Ca(Citrate)-       1.200e-006  9.559e-007    -5.921    -6.020    -0.099
             Mg(Citrate)-       4.557e-007  3.629e-007    -6.341    -6.440    -0.099
             CaH(Citrate)       2.639e-007  2.639e-007    -6.579    -6.579     0.000
             MgH(Citrate)       4.423e-008  4.423e-008    -7.354    -7.354     0.000
             Citrate-3          2.739e-008  5.556e-009    -7.562    -8.255    -0.693
             Fe(Citrate)-       8.946e-009  7.125e-009    -8.048    -8.147    -0.099
             CaH2(Citrate)+     3.770e-009  3.003e-009    -8.424    -8.522    -0.099
             H3(Citrate)        1.633e-009  1.633e-009    -8.787    -8.787     0.000
             MgH2(Citrate)+     1.255e-009  9.992e-010    -8.901    -9.000    -0.099
             FeH(Citrate)       1.030e-009  1.030e-009    -8.987    -8.987     0.000
             MnH(Citrate)       9.043e-010  9.043e-010    -9.044    -9.044     0.000
             K(Citrate)-2       8.604e-010  3.461e-010    -9.065    -9.461    -0.395
             Al(Citrate)        7.975e-010  7.975e-010    -9.098    -9.098     0.000
             Mn(Citrate)-       4.733e-010  3.770e-010    -9.325    -9.424    -0.099
             Fe(Citrate)        4.068e-011  4.068e-011   -10.391   -10.391     0.000
             NaH(Citrate)-      3.444e-011  2.743e-011   -10.463   -10.562    -0.099
             Na(Citrate)-2      2.253e-011  9.063e-012   -10.647   -11.043    -0.395
             AlH(Citrate)+      8.722e-012  6.946e-012   -11.059   -11.158    -0.099
             Al(Citrate)2-3     2.324e-012  2.996e-013   -11.634   -12.523    -0.890
             FeH(Citrate)+      1.170e-014  9.320e-015   -13.932   -14.031    -0.099
             Na2(Citrate)-      5.105e-015  4.066e-015   -14.292   -14.391    -0.099

Oxalate                       1.001e-002
             Oxalate-2          1.001e-002  4.925e-003    -2.000    -2.308    -0.308
             K(Oxalate)-        4.680e-006  3.920e-006    -5.330    -5.407    -0.077
             NH4(Oxalate)-      1.333e-006  1.117e-006    -5.875    -5.952    -0.077
             Al(Oxalate)+       3.222e-007  2.566e-007    -6.492    -6.591    -0.099
             Na(Oxalate)-       1.118e-007  9.365e-008    -6.952    -7.028    -0.077
             H2(Oxalate)        1.053e-008  1.053e-008    -7.978    -7.978     0.000
             Ca(Oxalate)        7.150e-009  7.213e-009    -8.146    -8.142     0.004
             Mg(Oxalate)        1.008e-009  1.017e-009    -8.997    -8.993     0.004
             H(Oxalate)-        3.659e-012  3.065e-012   -11.437   -11.514    -0.077
             Mn(Oxalate)        1.950e-012  1.967e-012   -11.710   -11.706     0.004
```

Appendix 6 (M2) Input data for PHREEQC simulation for mycorrhized petridish M2.

```
SOLUTION 1
        temp      23
        pH        5.33
        pe        4
        redox     pe
        units    mg/l
    K     234.77
    Ca    189.05
    Mg     43.29
    Na      4.15
    Mn      0.59
    Fe     10.06
    Al      0.009
    N     136.17 as NO3
    Cl      0.96
    S                  57.15 as SO4
    EDTA   51.90
    Oxalate 144.70 charge
END
```

----------Distribution of species----------

Species		Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate		1.001e-002				
	Oxalate-2	1.000e-002	4.924e-003	-2.000	-2.308	-0.308
	K(Oxalate)-	4.679e-006	3.919e-006	-5.330	-5.407	-0.077
	NH4(Oxalate)-	1.333e-006	1.116e-006	-5.875	-5.952	-0.077
	Al(Oxalate)+	3.218e-007	2.563e-007	-6.492	-6.591	-0.099
	Na(Oxalate)-	1.118e-007	9.364e-008	-6.952	-7.029	-0.077
	Ca(Oxalate)	7.151e-009	7.214e-009	-8.146	-8.142	0.004
	H2(Oxalate)	1.747e-009	1.747e-009	-8.758	-8.758	0.000
	Mg(Oxalate)	1.008e-009	1.017e-009	-8.996	-8.993	0.004
	Mn(Oxalate)	1.953e-012	1.970e-012	-11.709	-11.705	0.004
	H(Oxalate)-	1.490e-012	1.248e-012	-11.827	-11.904	-0.077

Appendix 6 (M3) Input data for PHREEQC simulation for mycorrhized petridish M3.

```
SOLUTION 1
          temp      23
          pH        6.45
          pe        4
          redox     pe
          units    mg/l
     K      234.77
     Ca     189.05
     Mg      43.29
     Na       4.15
     Mn                    0.59
     Fe      10.06
     Al                    0.009
     N      136.17 as NO3
     Cl       0.96
     S                   57.15 as SO4
     EDTA    51.90

     Oxalate            101.70 charge
     Citrate    0.08
 END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Citrate	4.234e-007				
Ca(Citrate)-	2.984e-007	2.376e-007	-6.525	-6.624	-0.099
Mg(Citrate)-	1.133e-007	9.021e-008	-6.946	-7.045	-0.099
Citrate-3	6.805e-009	1.381e-009	-8.167	-8.860	-0.693
Fe(Citrate)-	2.077e-009	1.654e-009	-8.683	-8.781	-0.099
CaH(Citrate)	2.028e-009	2.028e-009	-8.693	-8.693	0.000
MgH(Citrate)	3.398e-010	3.398e-010	-9.469	-9.469	0.000
K(Citrate)-2	2.138e-010	8.602e-011	-9.670	-10.065	-0.395
Al(Citrate)	1.695e-010	1.695e-010	-9.771	-9.771	0.000
Mn(Citrate)-	1.182e-010	9.413e-011	-9.927	-10.026	-0.099
Fe(Citrate)	9.445e-012	9.445e-012	-11.025	-11.025	0.000
FeH(Citrate)	7.389e-012	7.389e-012	-11.131	-11.131	0.000
MnH(Citrate)	6.978e-012	6.978e-012	-11.156	-11.156	0.000
Na(Citrate)-2	5.598e-012	2.252e-012	-11.252	-11.647	-0.395
CaH2(Citrate)+	8.950e-013	7.128e-013	-12.048	-12.147	-0.099
MgH2(Citrate)+	2.978e-013	2.372e-013	-12.526	-12.625	-0.099
NaH(Citrate)-	2.645e-013	2.106e-013	-12.578	-12.676	-0.099
Al(Citrate)2-3	1.228e-013	1.583e-014	-12.911	-13.801	-0.890
AlH(Citrate)+	5.730e-014	4.563e-014	-13.242	-13.341	-0.099
H3(Citrate)	1.198e-014	1.198e-014	-13.922	-13.922	0.000
Na2(Citrate)-	1.269e-015	1.010e-015	-14.897	-14.996	-0.099
FeH(Citrate)+	8.395e-017	6.686e-017	-16.076	-16.175	-0.099
Oxalate	9.999e-003				
Oxalate-2	9.992e-003	4.918e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.674e-006	3.915e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.329e-006	1.113e-006	-5.876	-5.953	-0.077
Al(Oxalate)+	2.753e-007	2.193e-007	-6.560	-6.659	-0.099
Na(Oxalate)-	1.117e-007	9.353e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.143e-009	7.206e-009	-8.146	-8.142	0.004
Mg(Oxalate)	1.007e-009	1.016e-009	-8.997	-8.993	0.004
H2(Oxalate)	1.004e-011	1.004e-011	-10.998	-10.998	0.000
Mn(Oxalate)	1.957e-012	1.974e-012	-11.708	-11.705	0.004
H(Oxalate)-	1.129e-013	9.459e-014	-12.947	-13.024	-0.077

Appendix 6 (M4) Input data for PHREEQC simulation for mycorrhized petridish M4.

```
SOLUTION 1
    temp      23
    pH        5.12
    pe        4
    redox     pe
    units    mg/l
K      234.77
Ca    189.05
Mg    43.29
Na     4.15
Mn     0.59
Fe    10.06
Al                0.009
N      136.17 as NO3
Cl     0.96
S       57.15 as SO4
EDTA    51.90

Oxalate 182.44 charge

END
```

----------Distribution of species----------

Species		Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.001e-002					
	Oxalate-2	1.001e-002	4.925e-003	-2.000	-2.308	-0.308
	K(Oxalate)-	4.680e-006	3.920e-006	-5.330	-5.407	-0.077
	NH4(Oxalate)-	1.333e-006	1.117e-006	-5.875	-5.952	-0.077
	Al(Oxalate)+	3.226e-007	2.569e-007	-6.491	-6.590	-0.099
	Na(Oxalate)-	1.118e-007	9.366e-008	-6.951	-7.028	-0.077
	Ca(Oxalate)	7.153e-009	7.215e-009	-8.146	-8.142	0.004
	H2(Oxalate)	4.596e-009	4.596e-009	-8.338	-8.338	0.000
	Mg(Oxalate)	1.008e-009	1.017e-009	-8.996	-8.993	0.004
	H(Oxalate)-	2.417e-012	2.025e-012	-11.617	-11.694	-0.077
	Mn(Oxalate)	1.952e-012	1.970e-012	-11.709	-11.706	0.004

Appendix 6 (M5) Input data for PHREEQC simulation for mycorrhized petridish M5.

```
SOLUTION 1
    temp      23
    pH        4.72
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na      4.15
Mn      0.59
Fe     10.06
Al      0.009
N       136.17 as NO3
Cl      0.96
S                  57.15 as SO4
EDTA   51.90

Oxalate 169.67 charge
Malate    0.16

END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Malate	1.213e-006				
Malate-2	1.178e-006	5.798e-007	-5.929	-6.237	-0.308
H(Malate)-	3.485e-008	2.775e-008	-7.458	-7.557	-0.099
H2(Malate)	2.362e-011	2.362e-011	-10.627	-10.627	0.000
Al(Malate)+	1.123e-011	8.945e-012	-10.950	-11.048	-0.099
Oxalate	1.002e-002				
Oxalate-2	1.001e-002	4.927e-003	-1.999	-2.307	-0.308
K(Oxalate)-	4.682e-006	3.922e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.334e-006	1.117e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.234e-007	2.575e-007	-6.490	-6.589	-0.099
Na(Oxalate)-	1.119e-007	9.370e-008	-6.951	-7.028	-0.077
H2(Oxalate)	2.901e-008	2.901e-008	-7.537	-7.537	0.000
Ca(Oxalate)	7.155e-009	7.218e-009	-8.145	-8.142	0.004
Mg(Oxalate)	1.009e-009	1.018e-009	-8.996	-8.992	0.004
H(Oxalate)-	6.075e-012	5.088e-012	-11.216	-11.293	-0.077
Mn(Oxalate)	1.950e-012	1.967e-012	-11.710	-11.706	0.004

Appendix 6 (M6) Input data for PHREEQC simulation for mycorrhized petridish M6.

```
SOLUTION 1
    temp      23
    pH        4.8
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na      4.15
Mn      0.59
Fe     10.06
Al                  0.009
N       136.17 as NO3
Cl      0.96
S                  57.15 as SO4
EDTA   51.90

Oxalate 161.30 charge
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.002e-002				
Oxalate-2	1.001e-002	4.927e-003	-1.999	-2.307	-0.308
K(Oxalate)-	4.682e-006	3.922e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.334e-006	1.117e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.233e-007	2.575e-007	-6.490	-6.589	-0.099
Na(Oxalate)-	1.119e-007	9.369e-008	-6.951	-7.028	-0.077
H2(Oxalate)	2.007e-008	2.007e-008	-7.697	-7.697	0.000
Ca(Oxalate)	7.155e-009	7.217e-009	-8.145	-8.142	0.004
Mg(Oxalate)	1.009e-009	1.017e-009	-8.996	-8.992	0.004
H(Oxalate)-	5.053e-012	4.232e-012	-11.296	-11.373	-0.077
Mn(Oxalate)	1.951e-012	1.968e-012	-11.710	-11.706	0.004

Appendix 6 (M7) Input data for PHREEQC simulation for mycorrhized petridish M7.

```
SOLUTION 1
    temp      23
    pH        5.36
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na      4.15
Mn      0.59
Fe     10.06
Al        0.009
N       136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate  90.32 charge
Malate    0.39
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Malate	2.957e-006				
Malate-2	2.937e-006	1.446e-006	-5.532	-5.840	-0.308
H(Malate)-	1.990e-008	1.585e-008	-7.701	-7.800	-0.099
Al(Malate)+	2.788e-011	2.220e-011	-10.555	-10.654	-0.099
H2(Malate)	3.091e-012	3.091e-012	-11.510	-11.510	0.000
Oxalate	1.001e-002				
Oxalate-2	1.000e-002	4.922e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.677e-006	3.918e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.332e-006	1.116e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.216e-007	2.561e-007	-6.493	-6.592	-0.099
Na(Oxalate)-	1.118e-007	9.360e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.148e-009	7.211e-009	-8.146	-8.142	0.004
H2(Oxalate)	1.521e-009	1.521e-009	-8.818	-8.818	0.000
Mg(Oxalate)	1.008e-009	1.017e-009	-8.997	-8.993	0.004
Mn(Oxalate)	1.953e-012	1.970e-012	-11.709	-11.706	0.004
H(Oxalate)-	1.390e-012	1.165e-012	-11.857	-11.934	-0.077

Appendix 6 (M8) Input data for PHREEQC simulation for mycorrhized petridish M8.

```
SOLUTION 1
    temp      23
    pH        5.43
    pe        4
    redox     pe
    units    mg/l
K       234.77
Ca      189.05
Mg       43.29
Na        4.15
Mn        0.59
Fe       10.06
Al          0.009
N       136.17 as NO3
Cl        0.96
S          57.15 as SO4
EDTA     51.90
Oxalate   70.84 charge
END
```

----------Distribution of species----------

	Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate			1.001e-002			
	Oxalate-2	1.000e-002	4.923e-003	-2.000	-2.308	-0.308
	K(Oxalate)-	4.678e-006	3.919e-006	-5.330	-5.407	-0.077
	NH4(Oxalate)-	1.332e-006	1.116e-006	-5.875	-5.952	-0.077
	Al(Oxalate)+	3.213e-007	2.559e-007	-6.493	-6.592	-0.099
	Na(Oxalate)-	1.118e-007	9.362e-008	-6.952	-7.029	-0.077
	Ca(Oxalate)	7.150e-009	7.213e-009	-8.146	-8.142	0.004
	H2(Oxalate)	1.102e-009	1.102e-009	-8.958	-8.958	0.000
	Mg(Oxalate)	1.008e-009	1.017e-009	-8.997	-8.993	0.004
	Mn(Oxalate)	1.954e-012	1.971e-012	-11.709	-11.705	0.004
	H(Oxalate)-	1.184e-012	9.914e-013	-11.927	-12.004	-0.077

Appendix 6 (M9) Input data for PHREEQC simulation for mycorrhized petridish M9.

```
SOLUTION 1
    temp      23
    pH        6.34
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na      4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate  26.64 charge
Malate   1.16
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Malate	8.795e-006				
Malate-2	8.788e-006	4.326e-006	-5.056	-5.364	-0.308
H(Malate)-	6.236e-009	4.967e-009	-8.205	-8.304	-0.099
Al(Malate)+	7.599e-011	6.052e-011	-10.119	-10.218	-0.099
H2(Malate)	1.014e-013	1.014e-013	-12.994	-12.994	0.000
Oxalate	9.992e-003				
Oxalate-2	9.985e-003	4.915e-003	-2.001	-2.308	-0.308
K(Oxalate)-	4.670e-006	3.912e-006	-5.331	-5.408	-0.077
NH4(Oxalate)-	1.329e-006	1.113e-006	-5.877	-5.953	-0.077
Al(Oxalate)+	2.926e-007	2.330e-007	-6.534	-6.633	-0.099
Na(Oxalate)-	1.116e-007	9.346e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.138e-009	7.201e-009	-8.146	-8.143	0.004
Mg(Oxalate)	1.006e-009	1.015e-009	-8.997	-8.993	0.004
H2(Oxalate)	1.665e-011	1.665e-011	-10.778	-10.778	0.000
Mn(Oxalate)	1.956e-012	1.973e-012	-11.709	-11.705	0.004
H(Oxalate)-	1.454e-013	1.218e-013	-12.838	-12.914	-0.077

Appendix 6 (M10) Input data for PHREEQC simulation for mycorrhized petridish M10.

```
SOLUTION 1
    temp      23
    pH        5.54
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg    43.29
Na                  4.15
Mn     0.59
Fe    10.06
Al                  0.009
N       136.17 as NO3
Cl      0.96
S                   57.15 as SO4
EDTA   51.90

Oxalate 204.96 charge
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.001e-002				
Oxalate-2	1.000e-002	4.923e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.679e-006	3.919e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.333e-006	1.116e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.205e-007	2.553e-007	-6.494	-6.593	-0.099
Na(Oxalate)-	1.118e-007	9.364e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.151e-009	7.214e-009	-8.146	-8.142	0.004
Mg(Oxalate)	1.008e-009	1.017e-009	-8.996	-8.993	0.004
H2(Oxalate)	6.641e-010	6.641e-010	-9.178	-9.178	0.000
Mn(Oxalate)	1.954e-012	1.972e-012	-11.709	-11.705	0.004
H(Oxalate)-	9.188e-013	7.696e-013	-12.037	-12.114	-0.077

Appendix 6 (M11) Input data for PHREEQC simulation for mycorrhized petridish M11.

```
SOLUTION 1
    temp      23
    pH        4.82
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate  131.38 charge
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.002e-002				
Oxalate-2	1.001e-002	4.926e-003	-2.000	-2.307	-0.308
K(Oxalate)-	4.682e-006	3.921e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.334e-006	1.117e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.233e-007	2.574e-007	-6.490	-6.589	-0.099
Na(Oxalate)-	1.119e-007	9.369e-008	-6.951	-7.028	-0.077
H2(Oxalate)	1.830e-008	1.830e-008	-7.737	-7.737	0.000
Ca(Oxalate)	7.154e-009	7.217e-009	-8.145	-8.142	0.004
Mg(Oxalate)	1.009e-009	1.017e-009	-8.996	-8.993	0.004
H(Oxalate)-	4.825e-012	4.041e-012	-11.316	-11.393	-0.077
Mn(Oxalate)	1.951e-012	1.968e-012	-11.710	-11.706	0.004

Appendix 6 (M12) Input data for PHREEQC simulation for mycorrhized petridish M12.

```
SOLUTION 1
    temp      23
    pH        4.83
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg    43.29
Na                4.15
Mn                0.59
Fe    10.06
Al       0.009
N      136.17 as NO3
Cl     0.96
S       57.15 as SO4
EDTA  51.90

Oxalate 122.93 charge
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.002e-002				
Oxalate-2	1.001e-002	4.926e-003	-2.000	-2.307	-0.308
K(Oxalate)-	4.682e-006	3.921e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.334e-006	1.117e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.232e-007	2.574e-007	-6.490	-6.589	-0.099
Na(Oxalate)-	1.119e-007	9.368e-008	-6.951	-7.028	-0.077
H2(Oxalate)	1.748e-008	1.748e-008	-7.757	-7.757	0.000
Ca(Oxalate)	7.154e-009	7.217e-009	-8.145	-8.142	0.004
Mg(Oxalate)	1.009e-009	1.017e-009	-8.996	-8.993	0.004
H(Oxalate)-	4.715e-012	3.949e-012	-11.326	-11.403	-0.077
Mn(Oxalate)	1.951e-012	1.968e-012	-11.710	-11.706	0.004

Appendix 6 (M13) Input data for PHREEQC simulation for mycorrhized petridish M13.

```
SOLUTION 1
    temp      23
    pH        6.87
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg    43.29
Na                  4.15
Mn                  0.59
Fe    10.06
Al                  0.009
N      136.17 as NO3
Cl     0.96
S       57.15 as SO4
EDTA  51.90

Oxalate 97.82 charge
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	9.986e-003				
Oxalate-2	9.980e-003	4.912e-003	-2.001	-2.309	-0.308
K(Oxalate)-	4.668e-006	3.910e-006	-5.331	-5.408	-0.077
NH4(Oxalate)-	1.324e-006	1.109e-006	-5.878	-5.955	-0.077
Na(Oxalate)-	1.115e-007	9.342e-008	-6.953	-7.030	-0.077
Al(Oxalate)+	8.271e-008	6.587e-008	-7.082	-7.181	-0.099
Ca(Oxalate)	7.135e-009	7.197e-009	-8.147	-8.143	0.004
Mg(Oxalate)	1.006e-009	1.015e-009	-8.997	-8.994	0.004
Mn(Oxalate)	1.927e-012	1.944e-012	-11.715	-11.711	0.004
H2(Oxalate)	1.450e-012	1.450e-012	-11.839	-11.839	0.000
H(Oxalate)-	4.288e-014	3.592e-014	-13.368	-13.445	-0.077

Appendix 6 (M14) Input data for PHREEQC simulation for mycorrhized petridish M14.

```
SOLUTION 1
    temp      23
    pH        5.92
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al        0.009
N     136.17 as NO3
Cl      0.96
S                  57.15 as SO4
EDTA   51.90

Oxalate  63.11 charge
Malate    0.51

END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Malate	3.867e-006				
Malate-2	3.859e-006	1.900e-006	-5.413	-5.721	-0.308
H(Malate)-	7.203e-009	5.737e-009	-8.142	-8.241	-0.099
Al(Malate)+	3.592e-011	2.861e-011	-10.445	-10.544	-0.099
H2(Malate)	3.081e-013	3.081e-013	-12.511	-12.511	0.000
Oxalate	1.000e-002				
Oxalate-2	9.995e-003	4.920e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.675e-006	3.916e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.331e-006	1.115e-006	-5.876	-5.953	-0.077
Al(Oxalate)+	3.152e-007	2.510e-007	-6.501	-6.600	-0.099
Na(Oxalate)-	1.117e-007	9.356e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.145e-009	7.208e-009	-8.146	-8.142	0.004
Mg(Oxalate)	1.007e-009	1.016e-009	-8.997	-8.993	0.004
H2(Oxalate)	1.153e-010	1.153e-010	-9.938	-9.938	0.000
Mn(Oxalate)	1.955e-012	1.973e-012	-11.709	-11.705	0.004
H(Oxalate)-	3.827e-013	3.206e-013	-12.417	-12.494	-0.077

Appendix 6 (M15) Input data for PHREEQC simulation for mycorrhized petridish M15.

```
SOLUTION 1
    temp      23
    pH        5.24
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                4.15
Mn                0.59
Fe    10.06
Al                0.009
N      136.17 as NO3
Cl     0.96
S                57.15 as SO4
EDTA  51.90

Oxalate 200.52 charge
Malate    0.53

END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Malate	4.019e-006				
Malate-2	3.983e-006	1.960e-006	-5.400	-5.708	-0.308
H(Malate)-	3.558e-008	2.834e-008	-7.449	-7.548	-0.099
Al(Malate)+	3.787e-011	3.016e-011	-10.422	-10.521	-0.099
H2(Malate)	7.284e-012	7.284e-012	-11.138	-11.138	0.000
Oxalate	1.001e-002				
Oxalate-2	1.000e-002	4.922e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.678e-006	3.918e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.332e-006	1.116e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.222e-007	2.566e-007	-6.492	-6.591	-0.099
Na(Oxalate)-	1.118e-007	9.362e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.149e-009	7.212e-009	-8.146	-8.142	0.004
H2(Oxalate)	2.643e-009	2.643e-009	-8.578	-8.578	0.000
Mg(Oxalate)	1.008e-009	1.017e-009	-8.997	-8.993	0.004
Mn(Oxalate)	1.952e-012	1.969e-012	-11.709	-11.706	0.004
H(Oxalate)-	1.833e-012	1.535e-012	-11.737	-11.814	-0.077

Appendix 6 (NM1) Input data for PHREEQC simulation for mycorrhized petridish NM1

```
SOLUTION 1
    temp      23
    pH        5.22
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate  93.25 charge
Citrate   0.20
END
```

----------Distribution of species----------

Species		Molality	Activity	Log Molality	Log Activity	Log Gamma
Citrate		1.058e-006				
	Ca(Citrate)-	6.835e-007	5.444e-007	-6.165	-6.264	-0.099
	Mg(Citrate)-	2.595e-007	2.066e-007	-6.586	-6.685	-0.099
	CaH(Citrate)	7.888e-008	7.888e-008	-7.103	-7.103	0.000
	Citrate-3	1.559e-008	3.164e-009	-7.807	-8.500	-0.693
	MgH(Citrate)	1.322e-008	1.322e-008	-7.879	-7.879	0.000
	Fe(Citrate)-	5.072e-009	4.039e-009	-8.295	-8.394	-0.099
	CaH2(Citrate)+	5.913e-010	4.710e-010	-9.228	-9.327	-0.099
	K(Citrate)-2	4.899e-010	1.971e-010	-9.310	-9.705	-0.395
	Al(Citrate)	4.536e-010	4.536e-010	-9.343	-9.343	0.000
	FeH(Citrate)	3.064e-010	3.064e-010	-9.514	-9.514	0.000
	MnH(Citrate)	2.705e-010	2.705e-010	-9.568	-9.568	0.000
	Mn(Citrate)-	2.698e-010	2.149e-010	-9.569	-9.668	-0.099
	MgH2(Citrate)+	1.968e-010	1.567e-010	-9.706	-9.805	-0.099
	H3(Citrate)	1.344e-010	1.344e-010	-9.872	-9.872	0.000
	Fe(Citrate)	2.306e-011	2.306e-011	-10.637	-10.637	0.000
	Na(Citrate)-2	1.283e-011	5.160e-012	-10.892	-11.287	-0.395
	NaH(Citrate)-	1.029e-011	8.196e-012	-10.988	-11.086	-0.099
	AlH(Citrate)+	2.603e-012	2.073e-012	-11.584	-11.683	-0.099
	Al(Citrate)2-3	7.526e-013	9.702e-014	-12.123	-13.013	-0.890
	FeH(Citrate)+	3.482e-015	2.773e-015	-14.458	-14.557	-0.099
	Na2(Citrate)-	2.906e-015	2.315e-015	-14.537	-14.635	-0.099
Oxalate		1.001e-002				
	Oxalate-2	1.000e-002	4.923e-003	-2.000	-2.308	-0.308
	K(Oxalate)-	4.679e-006	3.919e-006	-5.330	-5.407	-0.077
	NH4(Oxalate)-	1.333e-006	1.116e-006	-5.875	-5.952	-0.077
	Al(Oxalate)+	3.218e-007	2.563e-007	-6.492	-6.591	-0.099
	Na(Oxalate)-	1.118e-007	9.363e-008	-6.952	-7.029	-0.077
	Ca(Oxalate)	7.149e-009	7.212e-009	-8.146	-8.142	0.004
	H2(Oxalate)	2.899e-009	2.899e-009	-8.538	-8.538	0.000
	Mg(Oxalate)	1.008e-009	1.017e-009	-8.997	-8.993	0.004
	Mn(Oxalate)	1.952e-012	1.969e-012	-11.709	-11.706	0.004
	H(Oxalate)-	1.920e-012	1.608e-012	-11.717	-11.794	-0.077

Appendix 6 (NM2) Input data for PHREEQC simulation for mycorrhized petridish NM2.

```
SOLUTION 1
    temp      23
    pH        4.53
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate 176.87 charge
Citrate   0.06

END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Citrate	3.176e-007				
Ca(Citrate)-	1.493e-007	1.189e-007	-6.826	-6.925	-0.099
CaH(Citrate)	8.439e-008	8.439e-008	-7.074	-7.074	0.000
Mg(Citrate)-	5.669e-008	4.514e-008	-7.247	-7.345	-0.099
MgH(Citrate)	1.414e-008	1.414e-008	-7.850	-7.850	0.000
H3(Citrate)	3.450e-009	3.450e-009	-8.462	-8.462	0.000
Citrate-3	3.409e-009	6.911e-010	-8.467	-9.160	-0.693
CaH2(Citrate)+	3.099e-009	2.468e-009	-8.509	-8.608	-0.099
Fe(Citrate)-	1.133e-009	9.023e-010	-8.946	-9.045	-0.099
MgH2(Citrate)+	1.031e-009	8.212e-010	-8.987	-9.086	-0.099
FeH(Citrate)	3.353e-010	3.353e-010	-9.475	-9.475	0.000
MnH(Citrate)	2.885e-010	2.885e-010	-9.540	-9.540	0.000
K(Citrate)-2	1.071e-010	4.305e-011	-9.970	-10.366	-0.396
Al(Citrate)	9.948e-011	9.948e-011	-10.002	-10.002	0.000
Mn(Citrate)-	5.876e-011	4.679e-011	-10.231	-10.330	-0.099
NaH(Citrate)-	1.101e-011	8.769e-012	-10.958	-11.057	-0.099
Fe(Citrate)	5.152e-012	5.152e-012	-11.288	-11.288	0.000
Na(Citrate)-2	2.804e-012	1.127e-012	-11.552	-11.948	-0.396
AlH(Citrate)+	2.797e-012	2.227e-012	-11.553	-11.652	-0.099
Al(Citrate)2-3	3.611e-014	4.648e-015	-13.442	-14.333	-0.890
FeH(Citrate)+	3.810e-015	3.034e-015	-14.419	-14.518	-0.099
Na2(Citrate)-	6.350e-016	5.057e-016	-15.197	-15.296	-0.099
Oxalate	1.003e-002				
Oxalate-2	1.002e-002	4.930e-003	-1.999	-2.307	-0.308
K(Oxalate)-	4.685e-006	3.924e-006	-5.329	-5.406	-0.077
NH4(Oxalate)-	1.335e-006	1.118e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.235e-007	2.576e-007	-6.490	-6.589	-0.099
Na(Oxalate)-	1.119e-007	9.375e-008	-6.951	-7.028	-0.077
H2(Oxalate)	6.964e-008	6.964e-008	-7.157	-7.157	0.000
Ca(Oxalate)	7.158e-009	7.221e-009	-8.145	-8.141	0.004
Mg(Oxalate)	1.009e-009	1.018e-009	-8.996	-8.992	0.004
H(Oxalate)-	9.416e-012	7.886e-012	-11.026	-11.103	-0.077
Mn(Oxalate)	1.948e-012	1.965e-012	-11.710	-11.707	0.004

Appendix 6 (NM3) Input data for PHREEQC simulation for mycorrhized petridish NM3.

```
SOLUTION 1
    temp      23
    pH        5.43
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S                 57.15 as SO4
EDTA   51.90

Oxalate  143.80 charge

END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.001e-002				
Oxalate-2	1.000e-002	4.923e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.679e-006	3.919e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.333e-006	1.116e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.213e-007	2.559e-007	-6.493	-6.592	-0.099
Na(Oxalate)-	1.118e-007	9.363e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.151e-009	7.214e-009	-8.146	-8.142	0.004
H2(Oxalate)	1.102e-009	1.102e-009	-8.958	-8.958	0.000
Mg(Oxalate)	1.008e-009	1.017e-009	-8.996	-8.993	0.004
Mn(Oxalate)	1.954e-012	1.971e-012	-11.709	-11.705	0.004
H(Oxalate)-	1.184e-012	9.915e-013	-11.927	-12.004	-0.077

Appendix 6 (NM4) Input data for PHREEQC simulation for mycorrhized petridish NM4.

```
    SOLUTION 1
      temp      23
      pH        5.58
      pe        4
      redox     pe
      units    mg/l
    K     234.77
    Ca    189.05
    Mg     43.29
    Na                  4.15
    Mn                  0.59
    Fe     10.06
    Al                  0.009
    N     136.17 as NO3
    Cl      0.96
    S                  57.15 as SO4
    EDTA   51.90

    Oxalate  92.95 charge
    Malate    0.28

  END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Malate	2.123e-006				
Malate-2	2.114e-006	1.041e-006	-5.675	-5.983	-0.308
H(Malate)-	8.633e-009	6.876e-009	-8.064	-8.163	-0.099
Al(Malate)+	1.998e-011	1.591e-011	-10.699	-10.798	-0.099
H2(Malate)	8.078e-013	8.078e-013	-12.093	-12.093	0.000
Oxalate	1.001e-002				
Oxalate-2	1.000e-002	4.922e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.677e-006	3.918e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.332e-006	1.116e-006	-5.875	-5.952	-0.077
Al(Oxalate)+	3.201e-007	2.550e-007	-6.495	-6.594	-0.099
Na(Oxalate)-	1.117e-007	9.360e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.148e-009	7.211e-009	-8.146	-8.142	0.004
Mg(Oxalate)	1.008e-009	1.017e-009	-8.997	-8.993	0.004
H2(Oxalate)	5.522e-010	5.522e-010	-9.258	-9.258	0.000
Mn(Oxalate)	1.954e-012	1.971e-012	-11.709	-11.705	0.004
H(Oxalate)-	8.377e-013	7.017e-013	-12.077	-12.154	-0.077

Appendix 6 (NM5) Input data for PHREEQC simulation for mycorrhized petridish NM5.

```
SOLUTION 1
    temp      23
    pH        6.23
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al      0.009
N     136.17 as NO3
Cl      0.96
S                 57.15 as SO4
EDTA   51.90

Oxalate  111.34 charge

END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.000e-002				
Oxalate-2	9.997e-003	4.920e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.676e-006	3.916e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.331e-006	1.115e-006	-5.876	-5.953	-0.077
Al(Oxalate)+	3.027e-007	2.411e-007	-6.519	-6.618	-0.099
Na(Oxalate)-	1.117e-007	9.357e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.146e-009	7.209e-009	-8.146	-8.142	0.004
Mg(Oxalate)	1.007e-009	1.016e-009	-8.997	-8.993	0.004
H2(Oxalate)	2.767e-011	2.767e-011	-10.558	-10.558	0.000
Mn(Oxalate)	1.958e-012	1.975e-012	-11.708	-11.704	0.004
H(Oxalate)-	1.875e-013	1.570e-013	-12.727	-12.804	-0.077

Appendix 6 (NM6) Input data for PHREEQC simulation for mycorrhized petridish NM6.

```
SOLUTION 1
    temp      23
    pH        6.46
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al      0.009
N     136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate  96.74 charge
END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	9.999e-003				
Oxalate-2	9.993e-003	4.919e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.674e-006	3.915e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.329e-006	1.113e-006	-5.876	-5.953	-0.077
Al(Oxalate)+	2.734e-007	2.177e-007	-6.563	-6.662	-0.099
Na(Oxalate)-	1.117e-007	9.354e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.144e-009	7.206e-009	-8.146	-8.142	0.004
Mg(Oxalate)	1.007e-009	1.016e-009	-8.997	-8.993	0.004
H2(Oxalate)	9.590e-012	9.590e-012	-11.018	-11.018	0.000
Mn(Oxalate)	1.957e-012	1.974e-012	-11.708	-11.705	0.004
H(Oxalate)-	1.104e-013	9.243e-014	-12.957	-13.034	-0.077

Appendix 6 (NM7) Input data for PHREEQC simulation for mycorrhized petridish NM7.

```
SOLUTION 1
    temp      23
    pH        6.56
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn      0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate  85.28 charge

END
```

```
----------------------------Distribution of species-----------------------
                                                    Log       Log        Log
             Species           Molality    Activity  Molality  Activity     Gamma

Oxalate                    9.997e-003

             Oxalate-2        9.991e-003  4.917e-003    -2.000    -2.308    -0.308
             K(Oxalate)-      4.673e-006  3.914e-006    -5.330    -5.407    -0.077
             NH4(Oxalate)-    1.328e-006  1.113e-006    -5.877    -5.954    -0.077
             Al(Oxalate)+     2.454e-007  1.954e-007    -6.610    -6.709    -0.099
             Na(Oxalate)-     1.116e-007  9.351e-008    -6.952    -7.029    -0.077
             Ca(Oxalate)      7.142e-009  7.204e-009    -8.146    -8.142     0.004
             Mg(Oxalate)      1.007e-009  1.016e-009    -8.997    -8.993     0.004
             H2(Oxalate)      6.050e-012  6.050e-012   -11.218   -11.218     0.000
             Mn(Oxalate)      1.955e-012  1.972e-012   -11.709   -11.705     0.004
             H(Oxalate)-      8.764e-014  7.341e-014   -13.057   -13.134    -0.077
                    ---------------------- ----------------------
```

Appendix 6 (NM8) Input data for PHREEQC simulation for mycorrhized petridish NM8.

```
SOLUTION 1
    temp      23
    pH        5.91
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S        57.15 as SO4
EDTA   51.90

Oxalate  75.31 charge

END
```

----------Distribution of species----------

Species	Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.001e-002				
Oxalate-2	9.999e-003	4.922e-003	-2.000	-2.308	-0.308
K(Oxalate)-	4.677e-006	3.917e-006	-5.330	-5.407	-0.077
NH4(Oxalate)-	1.332e-006	1.115e-006	-5.876	-5.953	-0.077
Al(Oxalate)+	3.155e-007	2.512e-007	-6.501	-6.600	-0.099
Na(Oxalate)-	1.117e-007	9.360e-008	-6.952	-7.029	-0.077
Ca(Oxalate)	7.148e-009	7.211e-009	-8.146	-8.142	0.004
Mg(Oxalate)	1.008e-009	1.017e-009	-8.997	-8.993	0.004
H2(Oxalate)	1.208e-010	1.208e-010	-9.918	-9.918	0.000
Mn(Oxalate)	1.956e-012	1.973e-012	-11.709	-11.705	0.004
H(Oxalate)-	3.918e-013	3.282e-013	-12.407	-12.484	-0.077

Appendix 6 (NM9) Input data for PHREEQC simulation for mycorrhized petridish NM9.

```
SOLUTION 1
    temp      23
    pH        5.31
    pe        4
    redox     pe
    units    mg/l
K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S                  57.15 as SO4
EDTA   51.90

Oxalate  104.32 charge

END
```

```
-----------------------------Distribution of species-----------------------
                                                    Log       Log        Log
             Species           Molality    Activity Molality  Activity     Gamma

Oxalate                    1.001e-002

             Oxalate-2        1.000e-002  4.924e-003    -2.000    -2.308    -0.308
             K(Oxalate)-      4.679e-006  3.919e-006    -5.330    -5.407    -0.077
             NH4(Oxalate)-    1.333e-006  1.116e-006    -5.875    -5.952    -0.077
             Al(Oxalate)+     3.219e-007  2.564e-007    -6.492    -6.591    -0.099
             Na(Oxalate)-     1.118e-007  9.363e-008    -6.952    -7.029    -0.077
             Ca(Oxalate)      7.151e-009  7.214e-009    -8.146    -8.142     0.004
             H2(Oxalate)      1.916e-009  1.916e-009    -8.718    -8.718     0.000
             Mg(Oxalate)      1.008e-009  1.017e-009    -8.996    -8.993     0.004
             Mn(Oxalate)      1.953e-012  1.970e-012   -11.709   -11.705     0.004
             H(Oxalate)-      1.560e-012  1.307e-012   -11.807   -11.884    -0.077
```

Appendix 6 (NM10) Input data for PHREEQC simulation for mycorrhized petridish NM10.

```
SOLUTION 1
    temp      23
    pH        5.34
    pe        4
    redox     pe
    units    mg/l

K     234.77
Ca    189.05
Mg     43.29
Na                  4.15
Mn                  0.59
Fe     10.06
Al                  0.009
N     136.17 as NO3
Cl      0.96
S                  57.15 as SO4
EDTA   51.90

Oxalate  93.02 charge

END
```

----------Distribution of species----------

Species		Molality	Activity	Log Molality	Log Activity	Log Gamma
Oxalate	1.001e-002					
	Oxalate-2	1.000e-002	4.924e-003	-2.000	-2.308	-0.308
	K(Oxalate)-	4.679e-006	3.919e-006	-5.330	-5.407	-0.077
	NH4(Oxalate)-	1.333e-006	1.116e-006	-5.875	-5.952	-0.077
	Al(Oxalate)+	3.218e-007	2.562e-007	-6.492	-6.591	-0.099
	Na(Oxalate)-	1.118e-007	9.363e-008	-6.952	-7.029	-0.077
	Ca(Oxalate)	7.151e-009	7.213e-009	-8.146	-8.142	0.004
	H2(Oxalate)	1.668e-009	1.668e-009	-8.778	-8.778	0.000
	Mg(Oxalate)	1.008e-009	1.017e-009	-8.997	-8.993	0.004
	Mn(Oxalate)	1.953e-012	1.970e-012	-11.709	-11.705	0.004
	H(Oxalate)-	1.456e-012	1.220e-012	-11.837	-11.914	-0.077

Appendix 7. CANOCO software summary generated for interpreting PCA graphs.

```
Program CANOCO Version 4.53 June 2004 - written by Cajo J.F. Ter Braak
(C) 1988-2003 Biometris - quantitative methods in the life and earth
sciences
Plant Research International, Wageningen University and Research Centre
Box 100, 6700 AC Wageningen, The Netherlands
CANOCO performs (partial) (detrended) (canonical) correspondence analysis,
principal components analysis and redundancy analysis.
CANOCO is an extension of Cornell Ecology program DECORANA (Hill,1979)

For explanation of the input/output see the manual or
Ter Braak, C.J.F. (1995) Ordination. Chapter 5 in:
Data Analysis in Community and Landscape Ecology
Jongman, R.H.G., Ter Braak, C.J.F. and van Tongeren, O.F.R., Eds.
Cambridge University Press, Cambridge, UK, 91-173 pp.

*** Type of analysis ***
 Model             Gradient analysis
              indirect     direct     hybrid
linear         1=PCA       2= RDA       3
unimodal       4= CA       5= CCA       6
   ,,          7=DCA       8=DCCA       9
              10=non-standard analysis
Type analysis number
Answer =  1

Scaling of ordination scores         =    2
Diagnostics                          =    1

No samples omitted
Number of samples            25
Number of species            34
Number of occurrences       818


Centering/standardization by species  =    1
Centering/standardization by samples  =    0

No. of active  samples:     25
No. of passive samples:      0
No. of active  species:     34

Total sum of squares in species data =     58497.8
Total standard deviation in species data TAU =     8.29584

**** Summary ****

Axes                      1      2   3      4    Total variance
Eigenvalues:          0.669 0.196  0.097 0.021        1.000
Cumulative
Percentage
Variance of
species data:          66.9  86.5   96.2  98.3

Sum of all               eigenvalues           1.000

********************** End **********************
```